Synthesis of Quinone Methide Precursors and Derivatives to Resurrect Aged Acetylcholinesterase

Thesis

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By

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Abstract

Organophosphorus compounds (OPs) such as tabun, sarin and soman have been used as chemical warfare nerve agents. The advancements of chemical warfare agents used for military tactics exceed the research to reverse the toxic effects of the nerve agents. The demand to study OP nerve agents is crucial because of the damaging effects to people, the commercial availability, terrorist usage, and even the stockpiles in various countries. OP pesticides are also of significant concern as the largest number of deaths occurs due to exposure to such pesticides, especially in the third world. Exposure to OPs affects the central nervous system and causes a buildup of acetylcholine in the body by inhibiting acetylcholinesterase (AChE). The AChE is initially inhibited, followed by an aging process. There are known therapeutic oximes for reactivating the inhibited form of AChE; however, there are no known treatments for aged AChE. We are developing a library of quinone methide precursors (QMPs) to be used as potential re-alkylators in order to reverse the aging of AChE. These QMPs can be used to potentially re-alkylate the aged OP-AChE complex, followed by a subsequent reactivation step. This research is vital to enhance the pharmaceutical measures and further inspire more research done to counteract the aging process. Several frameworks were synthesized through synthetic routes including nucleophilic substitution, reductive amination, and Mannich reactions. We will present the synthesis of a small library of pyridine QMP frameworks and the screening of these compounds as re-alkylators and re-activators of both the inhibited and aged AChE (electric eel and human).

Dedicated to my family Barbara, Kutaiba, Serena, Lena, Suzanne, and Omar.

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Chapter 1. Introduction

The development of catastrophic weapons has been a common practice by various countries for centuries. For over 2,000 years, civilizations have been developing poisonous weapons to defend or conquer disputes.[12] More specifically, chemical warfare dates back as early as 600 B.C Greece, where the Athenians poisoned the Spartan's water supply of the city of Kirrha with a cardiac toxin derived from the Hellebore plant.[6] In counter tactics, the Spartans attempted to burn sulfur to create the first toxic smoke which was deployed on Athens.[12] Similarly, Genghis Khan attempted the same war tactic of burning sulfur pitch during the siege of fortified cities in 1200 A.D.[12] Over the centuries, armies continued to utilize chemicals such as the common war tactic of plant extracts to poison arrows in order to optimize their attacks and enemy causalities. Sadly, this was just the beginning of chemical warfare tactics that civilizations have used to attain power.

Advancements in science and chemistry have helped millions of people with the development of antibiotics in medicine; however, the scientific development of toxic chemicals has also harmed millions of people through advanced chemical warfare. Civilizations' natural tendencies are to attain power; therefore, they will utilize not only the bodies or strength of people but also their intelligence to develop weapons and chemicals to kill enemies in mass quantities while minimizing their own people's casualties. Bombs, missiles, and guns were not enough for some countries.[12] Sadly, countries developed methods to evoke emotional responses when killing mass amounts of people.[12] This led to chemical warfare because the chemicals are often invisible (gas) and people suffer a slow and agonizing death. Therefore, country's use of chemical warfare agents eliminated

the physical strength between two groups in war, which can be seen throughout history from the Americans in the American Civil War to the Germans in WWI.

In 1861, during the American Civil War, both armies utilized chemical weapons such as spraying chloroform, sulfuric acid, deploying poisonous gases (chlorine gas), and even filling grenades with arsenic-containing liquids.[6] In 1915 during WWI, the Germans dispatched over 170 metric tons of chlorine gas on the Allied troops.[17] As a result, more than 1,100 soldiers died and 7,000 were injured.[9] Throughout WWI, the Allies and the Germans used phosgene, diphosgene, mustard gas, hydrogen cyanide and cyanogen chloride (Figure 1.1).[9] Due to these extreme tactics, the United States opened a facility in Maryland to research chemical weapons. In total after WWI, 90,000 to 100,000 people died from chemical weapons, primarily due to poisonous gases.[17] These two wars were just the start of the 'chemists' war'. The power strike of countries blinded them to the devastation of delving into creating even more powerful chemical weapons without thinking about the counterpart of mass destruction. These countries deploy poisonous gases without containment or control over the span of area that a gas can cover, thus leading to millions of civilians and innocent people dying.

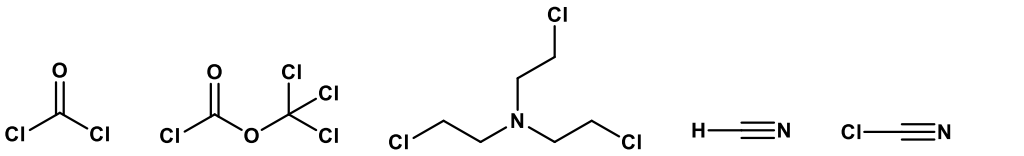


Figure 1.1. Chemical Warfare agents

The other side to this devastation is 'how do we prevent poisonous gas from killing thousands of soldiers and civilians?' During the chemist war in 1914-1918, the Allies

developed full body protection from the poisonous gases.[6] This included gas masks (Black Veil Respirator, reminiscent of bandit's mask) and fully-body canvas cloaks with transparent plastic viewing windows.[6] The filters in the gas mask either deactivated the poisonous air through contact with charcoal or trapped larger poisons as gas molecules with filters containing acid neutralizing or oxidizing agents.[6] Due to these advancements, casualties on both sides dwindled and yet again the two sides were at a stalemate until one side developed a more powerful chemical that penetrated the clothing and masks.

The allies and Germans utilized science, and more specifically advances in chemistry as a way to kill a large number of people versus ways to help or cure people. The stalemate ended in 1936 during the buildup to WWII, when a German chemist, Gerhard Schrader, was trying to synthesize a new pesticide to preserve food, but accidently created tabun, the first deadly organophosphorus compound (OPs) or nerve agent.[6] The Nazis manipulated his research from a pesticide to create three new chemical warfare agents: tabun, sarin and soman.[7] These three nerve agents are commonly known as the G agents: GA (tabun), GB (sarin) and GD (soman), refer to Figure 1.2, which were named after Gerhard Schrader.

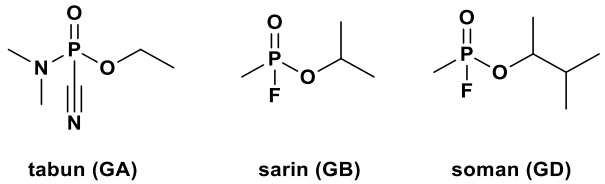


Figure 1.2. Common OP compound's structures used for both chemical warfare and pesticides

During WWII, Schrader furthered his research and developed a series of fluorine containing esters such as di-isopropyl fluorophosphate (DFP) and sarin, pyrophosphate esters including TEPP and octamethylpyrophosphortetramide (OMPA), and thio- and

thionophosphorus esters including parathion and its oxygen analog paraxon.[16] The pharmacological and toxicological studies of the Schrader's compounds were tested and the results showed that the toxicity and potency of the chemicals prevented them to be used for insecticides. However, these compounds were considered as potential chemical warfare agents. The Germans stockpiled tons of nerve agents which were never deployed on the Allies, but some poisonous gases were used in concentration camps. The stock piles include a total of 12,000 tons of tabun and more than 50,000 tons organic phosphorous compounds have been developed.[16] After WWII, researchers from both sides shared their knowledge and mechanistic effects of the nerve agents. This led to a new series of more stable nerve agents known as the V agents – refer to Figure 1.3. V agents are sulfur-containing agents and are approximately ten-fold more toxic than sarin.[10] Members of the V series were initially synthesized by the British government.[16] In addition, the United States mass produced 4400 tons of VX from 1961 to 1968.[16]

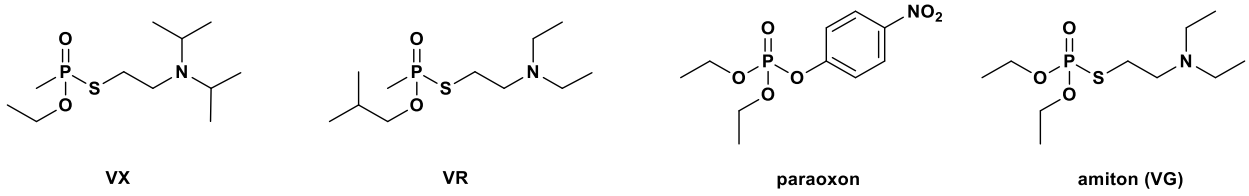


Figure 1.3. V series and pesticides

Some of these OPs have been used in recent times; during the Iran and Iraq war, different OP nerve agents were dispatched by the Iraqis against the Iranians. The Iraqi forces proceeded to attack the Kurdish city of Halabjah, deploying both sarin and tabun.[1] Due to the lack of containment or controlled deployment to a specific enemy area, the attack killed 5,000-8,000 people including soldiers and civilians.[16]

Aside from using chemical warfare agents as war tactics, if these nerve agents were to get in the wrong hands, it could lead to chemical terrorism. The first reported chemical terrorism was in 1994 by the Au, Shinrikyo Cult.[16] This cult deployed sarin gas in public places. Shortly after, in 1995, nerve gas, more specifically sarin gas, was deployed in a Tokyo subway station by terrorists, killing 13 people and several thousand people were injured.[16] In 2013, hundreds of Syrian civilians were killed by sarin gas from their own government.[11]

The usage of Organophosphorus compounds is not limited to chemical warfare and terrorism alone but also commonly used as pesticides and herbicides. Less lethal OPs are used for pest and disease control for farmer's crops. These OPs include malaoxon, paraoxon and chlorpyrifos oxon currently used in agriculture.[16] Even though, there has been some improvements of these OP pesticides toxicity, small residues of OP toxin can remain in the vegetation and individuals can ingest these toxic crops as well as direct OP exposure to agricultural workers. In developing countries, agricultural workers reported annual incidences of acute pesticide poisoning were (sadly) 18.2 per 100,000 full time workers.[16] Today, it is estimated that 3 million people are exposed to OP pesticide compounds worldwide, resulting in, on average, 300,000 deaths annually.[16]

Unfortunately, the antidotes or countermeasures for both OP pesticides and chemical warfare have not had such an illustrious history and are rather lacking. The advancements of chemical warfare agents used for military tactics exceed the research to inhibit the effects of the nerve agents.

The demand to study OP nerve agents is crucial because of the damaging effects to people, the ease of commercial availability and even the stockpiles in countries waiting

to be deployed in case of a terrorist attack. Even more alarming, sarin gas is so highly toxic that a single drop of sarin the size of a pin or 800 micrograms is enough to kill a single human and most times victims will die in a few seconds.[13]

Such OP nerve agents are toxic phosphoryl groups that covalently inhibit the enzyme, acetylcholinesterase (AChE). Acetylcholinesterase is an enzyme found in the central nervous system and peripheral nervous system that regulates the concentration of acetylcholine (ACh) found in the neuromuscular junction by hydrolyzing the ACh neurotransmitter. ACh is essential in both the central nervous system and peripheral system. In the peripheral nervous system, the major role of ACh is to transmit a signal between motor nerve and skeletal muscle to trigger contractions and to move the human body.[8] Its critical role in the CNS is as a modulator that activates different areas of the brain that control attention, memory, arousal and motivation. Due to its essential role in the human body, it is essential that the amount of ACh present be strictly governed by enzymes. More specifically, AChE regulates acetylcholine by hydrolyzing 5000/25000 acetylcholine per second into choline and acetic acid by the catalytic triad of the active site and the oxyanion hole.[15]

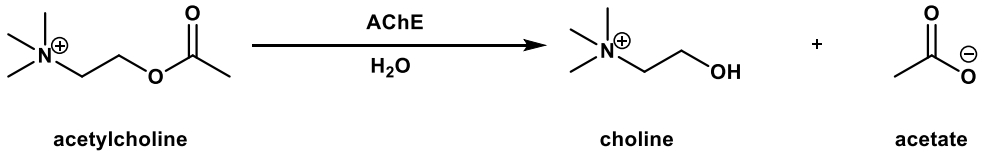
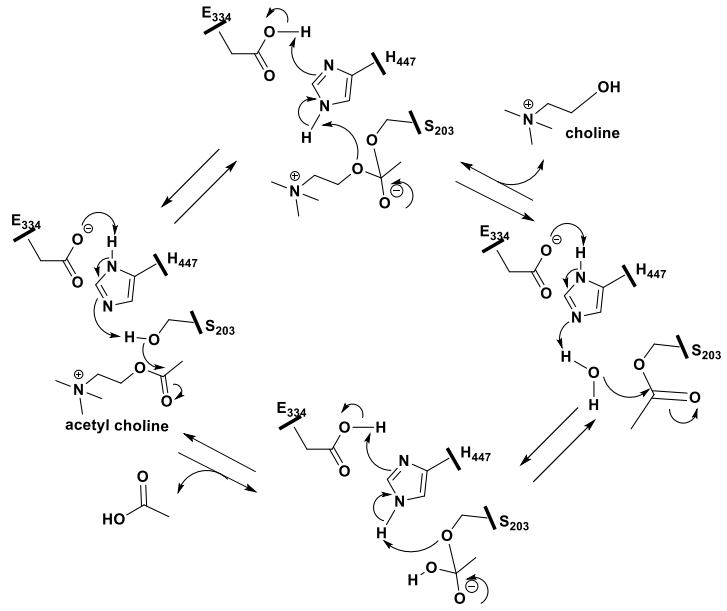


Figure 1.4. Acetylcholine hydrolysis

The catalytic triad is composed of three key amino acid residues Ser-203, His-447 and Glu-334, and similar to serine proteases found throughout the body.[4] The catalytic process starts by the activated Ser-203 nucleophilic attacking the carbonyl carbon of the

ACh (Scheme 1.1).[4] However, after exposure to the OP compound that is a substrate analogue to ACh, the phosphoryl center covalently bonds to the serine residue of the active site, which inhibits the enzyme and leads to the accumulation of ACh in the neurosynaptic junction (Scheme 1.2).[20] An overabundance of ACh can lead to adverse effects such as continuous stimulation of muscles, glands and stimulation of the CNS. Less severe symptoms include muscle weakness, disorientation and reduced vision.[14] More severe poisoning symptoms include respiratory failure, convulsions, paralysis, vomiting, and even death.[14] The severity of these OP gases is devastating – only 5 tons of sarin at the right concentration would be enough to completely aboliterate all of humanity.



Scheme 1.1. The hydrolysis of acetylcholine into choline

The flip side to this devastation is how do we reverse the effects of the OP agents or prevent the ingestion of the poisonous compounds. The first attempts started with gas masks and full body cloaks or ghost costumes; however, such countermeasures only

worked against tear gas, chlorine and phosgene/diphosgene. Mustard gas and the nerve agents created after WWI could penetrate the soldiers' protection gear. Therefore, another countermeasure must be taken to prevent these devastating fatalities moving forward. In 1951, pharmacological treatment studies hoped to reverse the effects of OP compounds.[2] There are three pharmacologic treatments that are commonly administered: anticholinergic drug (atropine), anticonvulsant drugs (benzodiazepines), and pyridinium oximes (Figure 1.5).

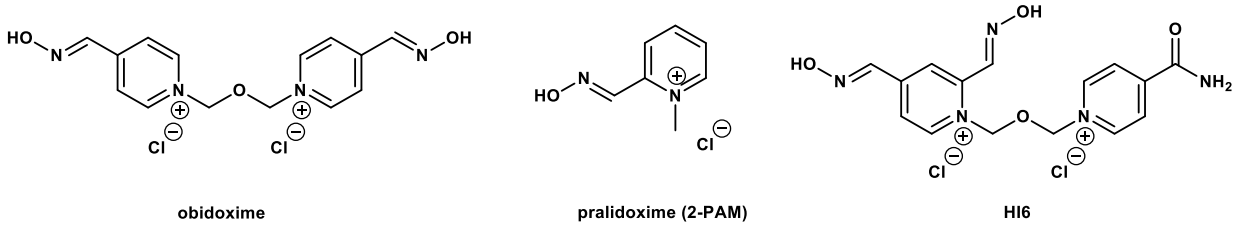
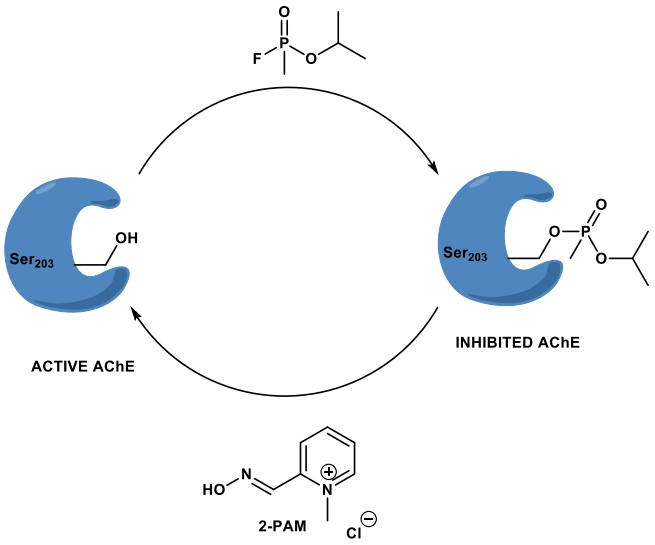


Figure 1.5. Current accepted treatments to reverse OP exposure before enzyme is aged.

The first pharmacological treatment was atropine for their parasympathomimetic effects.[2] The anticonvulsant, benzodiazepine, limits the amount of ACh released in the synaptic cleft; therefore, the patient will be less susceptible to convulsions. With the combination of benzodiazepine with atropine or pyridinium oximes,[19] benzodiazepine with atropine will combat both the physical symptoms (convulsions and muscle fasciculations) and the chemical problem by reactivating the AChE enzymes.[2] Pyridinium oximes, such as 2-PAM (Figure 1.5), reactivate the OP-inhibited AChE by nucleophilic attack, thereby releasing the phosphonate as a leaving group (Scheme 1.2). This regenerates the native serine residue, which then can hydrolyze ACh again.



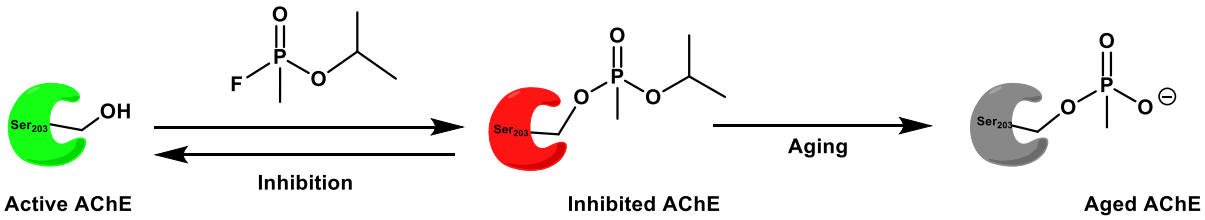
Scheme 1.2. Organophosphorus inhibition of AChE

However, there are various problems with the use of current pyridinium oximes, such as 2-PAM, and atropine. First, the dosage of the amount of oxime administered to the patient is dependent on the specific OP inhibiting the active site. The amount and the structure of the oxime mediates its effectiveness for a specific OP, and fatal consequences can occur if applied incorrectly. Moroever, and leading to the second major problem, there is a second step in the OP interaction with AChE. The inactive and inhibited phosphylated serine can be irreversibly dealkylated into the "aged" OP-AChE complex (Scheme 1.3). This aged complex can not be reactivated by pyridinium oximes. Nearby amino acids provide an oxyanion hole that stabilizes the phosphylated anionic form of the OP-AChE complex, making it more favorable in this irreversible form.[7] The time period for aging to occur depends on the OP inhibiting the enzyme and Table 1.1 provides some half times to aging for different OP compounds.[20] Lastly, all of the currently accepted treatments for reactivators are positively charged, such as pyridinium oximes; consequently, the drug can not pass the blood brain barrier to reactivate the AChE in the

CNS. This will cause a buildup of ACh in the brain leading to fatal effects. Currently, there is a deficit of pharmaceutical measures to medically counteract the aging process from OP exposure.

Organophosphorus Compound	**Aging time**
Soman	2 minutes
Sarin	5 hours
Cyclosarin	22 hours
VX/tabun	>40 hours

Table 1.1. Rates of aging for different OP compounds[20]



Scheme 1.3: Process of reversible inhibition and irreversible aging of AChE by sarin.

In the different research efforts for countermeasures against OP poisoning, there have been four focused research efforts to treat OP-aged AChE. First, one effort includes slowing down the aging process. The key mechanism of this solution is to have a drug that slows down the aging process; thereby, allowing more time for other drugs to reactivate the inhibited AChE before it becomes aged. In 1996, Berry and Davies were the first to successfully prove the capability of effectors to slow down the aging process. They reported 2-40 mM *N*-methylpyridinium iodide lowered the aging rate of soman-inhibited AChE by 33-69% and cyclosarin-inhibited AChE by 52-100%.[19] In 1970, Crone reported results for how six quaternary ammonium compounds affect the aging kinetics of sarin, and from that work, one effector slowed down the aging rate by 77%.[19] In addition, van Dongen *et al.* tested atropine, a common treatment for OP poisoning, slowed down the aging of sarin-inhibited rat and human AChE by 28-60%, but at a dose

of 1 mM.[19] This dosage of atropine is orders of magnitude higher than the clinical dosage of atropine, so these effects are never seen, thus leading to the key reason that this method of treatment is not currently used. The effectors must be administered at a high mM concentration, which is too high for clinical treatment and creating other toxic issues for humans. In addition, the main purpose of the effectors is to slowdown the aging process; therefore, the root problem of OP inhibition is still there. Once all of the AChE becomes aged, the effectors will have no impact and the problem to resurrect or realkylate the AChE is still in play.

The second and third method includes up-regulating the expression of AChE and introducing exogenous AChE. These methods both showed the newly synthesized AChE and exogenous AChE can replace any impaired AChE (inhibited or aged), therefore, recovering the AChE activity in the victim.[19] However, both strategies cannot resurrect aged AChE; thus, the problem of resurrecting the aged AChE is still relevant.

Lastly, the attempt to realkylate the aged AChE has offered the most promising solution to undo the aging process. Although many organic molecules can be synthesized, it is critical for one specific molecule to have the correct reactivity and orientation to re-alkylate the aged AChE. More specifically, the goal is to reverse the dealkylation of the oxyanion of the phosphyl group; therefore, the negatively charged phosphyl group will be neutralized after re-alkylation. This solution paired with reactivation drugs such as 2-PAM will return the AChE to its native form. Across the decades, various types of organic molecules as electrophilic molecules have been tested to re-alkylate aged AChE.

Sulfonate molecules were the first molecules to be tested to re-alklate aged AChE because they are good leaving groups. In 1969, Blumbergs *et al.* synthesized a library of sulfonic esters.[19] The year after, α-haloketones were evaluated for a solution because of the increased electrophilicity of the α-carbon.[19] Steinberg *et al.* made a library of haloketones consisting of 20 alkylating agents.[19] More specifically a bromoketone was experimentally proven to realkylate a model compound as a methylphosphonate; however, Steinberg was unsuccessful in demonstrating any realkylation of *in vitro* aged AChE.[19] In 2013, a series of methoxypyridinium derivatives were evaluated for their ability to re-alkylate aged AChE by transferring methyl groups to the nucleophilic oxygen.[19] Two reaction mechanisms were proposed, as shown in Figure 1.6. However, there have not been any reports of the success of these molecules for their alkylation ability in an *in vitro* experiment with aged AChE.

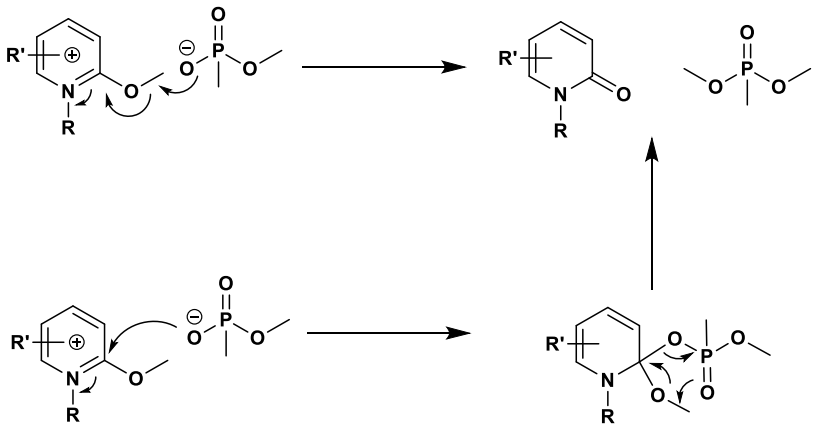


Figure 1.6. Quinn *et al.* proposed two possible mechanisms of methyl transfers for realkylation[19]

Similarly, sulfoniums were evaluated for their capabilities in biological transmethylation also known as (*S*)-adenosyl methionine.[19] This study made a breakthrough in demonstrating the orientation that the drug enters the active site is

critical. In this study, the researchers used a force field to 'pull' the compound out of the active site and, and the orientation of the compound flips as it enters or leaves the active site. This gives the compound the ability to react with the active site in all orientations instead of the its original docking in the active site. Their mechanistic capabilities are shown in Figure 1.5. These molecules were never tested for their alkylation ability.

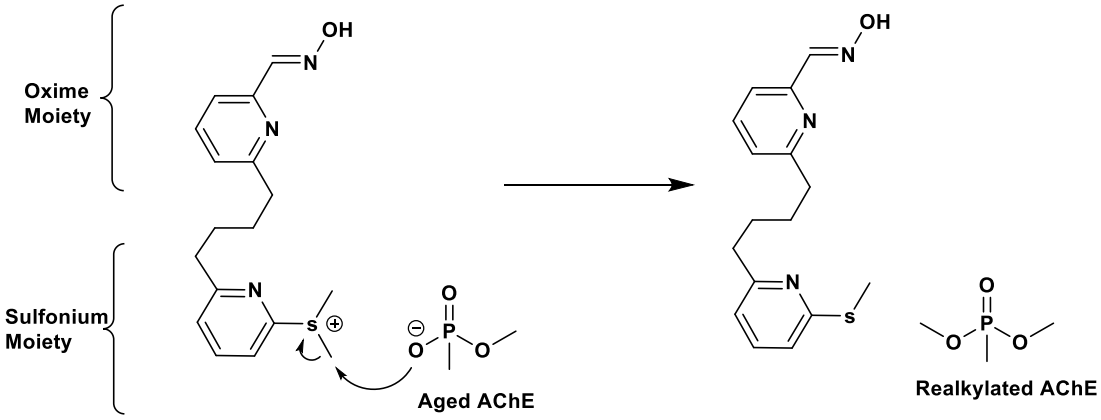


Figure 1.7. Chandar et al purposed methyl transfer reaction with sulfonium compound[19]

A promising family of organic electrophiles are quinone methides (QM) and quinone methide precursors (QMP). In 2004, Zhou *et al.* showed that quinone methides (QM) have the ability to alkylate different amino acids *in vitro*.[18] These quinone methide precursors mimic edrophonium, an oxyanilinium-based inhibitor of AChE that binds to the active site.[5] The QM were synthesized from quinone methide precursors (QMP) via thermal or photochemical means. Many modification and attachments on the aromatic ring have different reactivity properties.[5] One initial family of compounds included the *para*-substituted QMPs. In addition, Bakke *et al.* showed the QMs alkylating properties;

however, the QMs were produced from the oxidation reaction with lead oxide and silver oxide from QMPs versus the thermal means (Figure 1.8).[3]

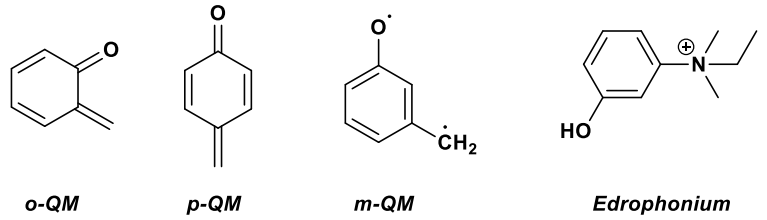


Figure 1.8. General quinone methide structures and the structure of edrophonium.

The synthesized QMs were found to have re-alkylated a phosphodiester bond and lactonized to form a trialkyl phosphate under aqueous conditions.[3] Although QMs have been shown applied as electrophiles in different applications, none of them have been studied as re-alkylators for aged AChE.

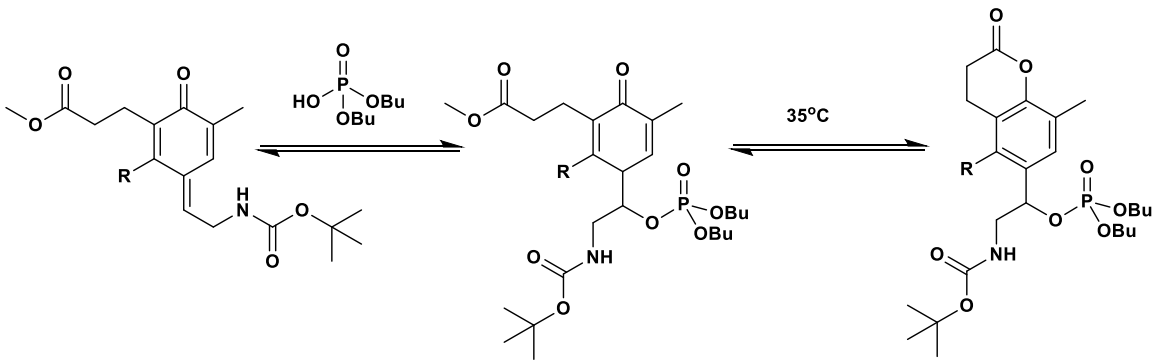
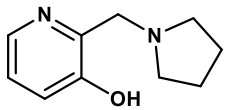


Figure 1.9. QM alkylates a phosphodiester bond via lactonization[3]

However, both studies with QMPs gave way for inspiration and were vital starting point for our library of potential resurrectors of aged AChE activity. Using computational chemistry, the possible options of QMPs were narrowed down because these methods were able to determine the optimal QMPs that had potential reactivity and potential orientational preference in the enzyme's active site.

While our lab continues to investigate the mechanism, we have found compounds that resurrect aged AChE. We hypothesized that these compounds re-alkylate aged AChE followed by a reactivation step to produce active uninhibited AChE. One of the most successful resurrectors in our lab is shown in Figure 1.10. The objective of my research is to explore two key questions and to aid in our understanding of their mode of action. The questions include: 'how different amines substitution will affect resurrection', 'how blocking the phenolic oxygen will affect resurrection', and 'how adding a substituent on the benzylic carbon will affect resurrection'. This report will unfold the process of answering these questions by first synthesizing, purifying, and screening the organic molecules for resurrection.



1.1

Figure 1.10. One of our most successful resurrectors: 2-(pyrrolidine-1-ylmethly)pyridine-3-ol.

In summary, organophosphorus compounds inhibit human AChE, which may cause fatal effects on humans. Current treatments, such as 2-PAM, have proven to reverse the inhibition processes; however, limitations with these drugs include its ability to pass the blood brain barrier, optimal dosage, and correct time frame of dosage administration. The major concern is the time frame of administration because if an oxime is not given at the right time, the AChE becomes aged or the "OP-AChE" complex dealkylates (Figure 1.11). Current standards of care cannot resurrect this irreversible aging process. Resurrection is the process of a compound's ability to re-alkylate the aged enzyme, followed by reactivation to produce active AChE. Therefore, this research aims

to synthesize quinone methide precursors to be used as potential re-alkylators of aged AChE, followed by reactivation with current treatments (2-PAM) or a different reactivator. In addition, this research is vital to enhance the pharmaceutical measures and further inspire more research to be done to medically counteract OP exposure.

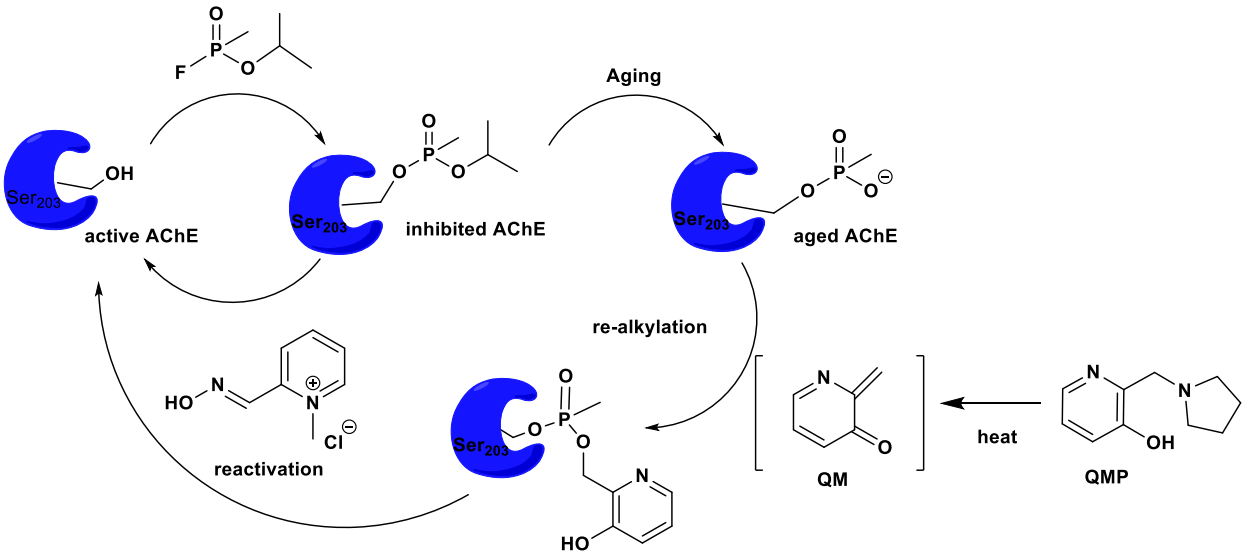


Figure 1.11. Research Significance

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Chapter 2. Synthesis of Organic Molecules

Introduction

The rapid rate of new and evolving diseases and illnesses is becoming a serious problem due to the treatments and drugs not being able to keep up with these devastating elements. However, synthetic chemistry or drug development has helped to hinder the spread of disease.[4] Organic synthesis paved the way to produce important compounds that save and improve millions of people's lives by providing small drug-like materials that can eradicate illnesses and diseases.[4]

The start of total synthesis began in 1828 of urea by Wohler (Figure 2.1).[4] This major breakthrough not only was the first documented total synthesis but was the first time an organic molecule was made into an inorganic molecule. This major stepping stone in total synthesis paved the way for the tools to design natural products, conformational information and structure, and discovery of new biologically important molecules.

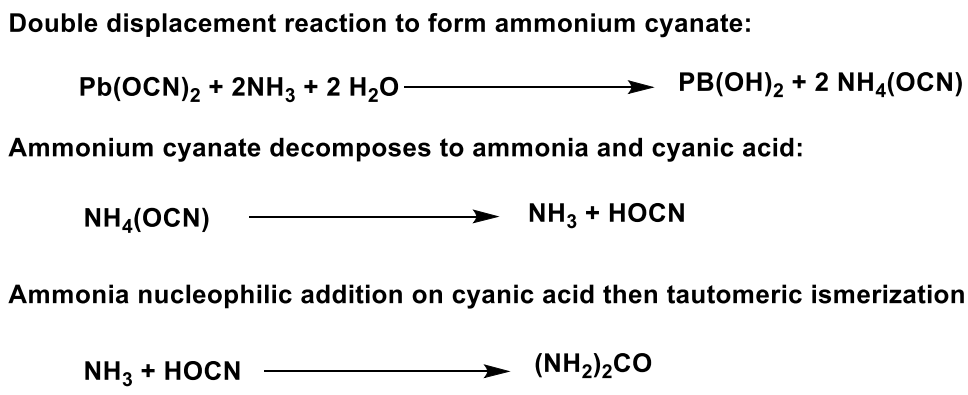


Figure 2.1. Synthesis of Urea

This led to the realization that natural products can be constructed and manipulated to enhance and optimize its curing properties of diseases and illnesses through total synthesis. Total synthesis is a complex and arduous process to complete for complex molecules.[4] For these procedures, the atoms must be constructed in a specific orientation and position throughout the reaction scheme. In addition, the order of the reaction steps to which each piece of a molecule can be assembled and constructed without affecting a different part of the same molecule.[4,15] Therefore, not only closely monitoring the steps of the reactions are important, but also the types of reagents, temperature and environment conditions. These are all factors that must be regulated to construct the correct molecule and to optimize the final yield of a target compound through total synthesis.[4]

Initially, the synthesis of molecules had little innovation or analysis required to synthesize the target compounds because the desired compound closely resembled the starting materials. As breakthroughs in chemistry, biochemistry, biology, and drug synthesis unfolded, it gave rise for a new era of retrosynthetic analysis.[16] This entails synthesizing more complicated molecules from simple readily available starting material. This era began in post-World War II due to the breakthrough of five key advancements in synthesis:[4]

1. Construction of detailed mechanisms for a host of organic reactions
2. Introductions of conformation analysis and stereo-chemical studies
3. New spectroscopic tools for structural analysis
4. Use of chromatography for analysis and separation
5. Discovery of new selective chemical reagents

This new era of total synthesis was driven by a chemist, R. B. Woodward, who contributed to designing natural products such as strychnine, reserpine, and Vitamin B_{12} (Figure 2.2).[4] More specifically, the novel syntheses were noted by utilizing rings to control stereochemical centers. Woodward's synthesis pathways for his three key target compounds starting the building blocks of these complicated synthetic routes.[4] The synthesis of strychnine was the first alkaloid to be isolated. It was said that Woodward's synthesis "[it] ushered in a golden era of total synthesis and installed unprecedented confidence in, and respect for, the science of organic synthesis."[17] Woodward's second major contribution was his synthesis of reserpine through his ability to control stereochemistry by using unique ring properties to create it. In 1952, it was proven to have vital medicinal properties that include treating hypertension, nervous and mental disorders.[4] Lastly, his total synthesis of Vitamin B_{12} contributed to organic chemistry by the development of a new synthetic reaction to from a carbon-carbon bond.[4] Woodward's contribution was just the start to retrosynthetic synthesis, but it has been the gateway to forever change the chemist's approach to total synthesis and to allow for complicated construction of target molecules from simple starting materials to enhance the drug's potency in the body.

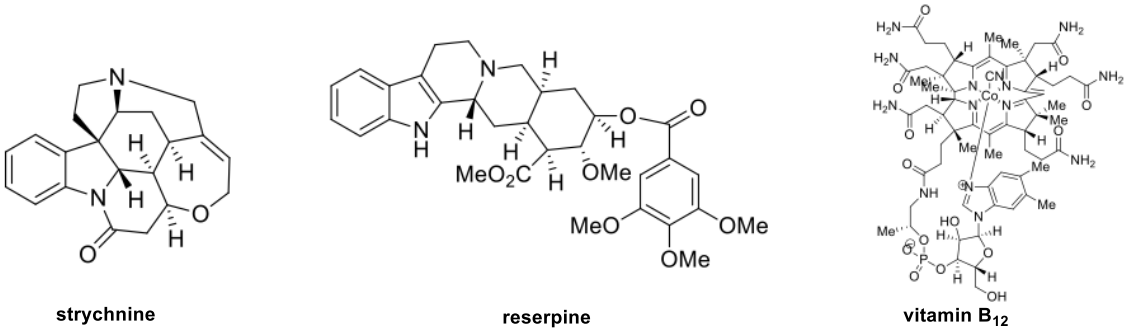


Figure 2.2. Woodward's natural compounds developed by total synthesis

After realizing chemists have the capabilities to produce natural compounds, they used the techniques to synthetically make organic compounds. The new era of synthetic drugs or compounds chemically developed not naturally found in nature began in the 1900s and continue today.[16] This novel technique has not only helped recreate scarce natural compounds in mass quantities but also optimizing the activity of the known drugs and enhancing and developing new derivatives of drugs as bacteria and virus become resistant to the previous drugs.[12]

For example, chemical synthesis played a major role in eradicating malaria in tropical regions. The cinchona tree bark contained the natural cure to this disease.[12] More specifically, scientists isolated quinine from the bark, which had the healing properties.[12] Sadly, the cinchona trees began to become extinct; therefore by 1932, only 13% of the world's demand could be supplied.[12] Through organic synthesis, German scientists constructed quinine substitutes that had the same antimalarial properties.[12] The total synthetic synthesis played a huge role in developing new antimalarial drugs in large quantities from easily available chemicals. Taxol is an anticancer drug that was naturally derived from yew root synthetic methods were developed to allow for its production.[12] This paved a new era of readily producing large quantities of natural compounds or derivatives of natural compounds through synthetic approach to save thousands of lives from illnesses and diseases.

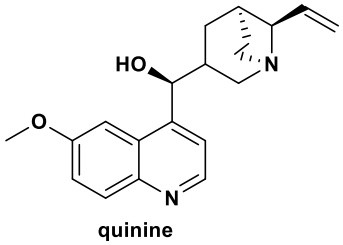


Figure 2.3. Malaria treatment with quinine

In addition, synthetic chemistry provided a solution to bacteria becoming drug resistant to natural antibiotics by developing analogs to the natural antibiotics.[9] The major contribution to synthetic antimicrobial drugs was Gerhard Domagk.[9] He discovered the antibacterial activity of synthetic dye, prontosil – more specifically the sulfanilamide (active breakdown product of prontosil) that could treat streptococcal infections.[9] His own daughter was the first human to take the drug.[9] The drug completely cured her.[9] This was the first synthetic antimicrobial created and served as a stepping stone for the discovery and production of additional classes of not only synthetic antimicrobials but also other classes of drugs. Synthetic chemistry offers hope to diseases that do not have therapeutic cure by the ability to manipulate and construct any type of molecule.

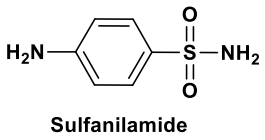


Figure 2.4. Structure of Sulfanilamide

Due to the breakthrough in biology providing detailed information at the molecular level, drugs have been able to be enhanced through refining the structure, developing new structure, or optimizing the known drug activity.[16] For example, analogues of an original drug such as changing a functional group from methyl, ethyl, or isopropyl have enhanced the drug's purpose.[16] Therefore, synthetic chemistry entails not only total synthesis or constructing a molecule but also partial modifications to the molecules.

The ability to modify the functional groups and the structure of drug molecules gives us the capability to harness the desired pharmacodynamic and pharmacokinetic effects.[8] The functional groups on a drug molecule are critical for either enhance the drugs activity or hinder it. For example the addition or absences of a methyl group or other alkyl chain on a drug structure can effect drug's selectiveness in the active site, its durations and its route of administration.[8] The alterations could also enhance the activity of the drug, increase absorption, decrease adverse effects, or provide other therapeutic benefits.[8] Changing the functional groups affects the overall effectiveness of a drug due to three main characteristics of functional groups: electronic effects, solubility and steric effects.[1] For electronic effects, functional groups can either be electronic donating groups or electronic withdrawing.[1] If the group is electron donating, the it can increase the nucleophilicity of a group. These molecules either contain negative charge or a lone pair of electrons that can used to form covalent bonds. In contrast, electron withdrawing groups, through induction, pull electron density away from nearby atoms.[1] Electron withdrawing groups can serve as electrophilic groups or 'electron loving'. The manipulation of the drug's functional groups can have a critical impact on its effectiveness in the body and the target enzyme's active site.

In addition, functional groups dictate the drugs ability to be water or lipid soluble based on the polarity, acidic and basic of the functional groups.[1] More specifically, the functional groups ability to form hydrogen bonds increases its water solubility and hydrophobic or nonpolar functional groups tend to be lipid soluble. Lastly, steric effects can have a huge impact on the ability of the drug to fit in relevant binding sites. The size of the functional groups has a direct impact on the drug's selectivity, binding interaction, and rate of metabolism or degradation.[1] In total, the ability to change the structure of a drug through addition or absences of functional groups has been proven to effect both its pharmacodynamic and pharmacokinetic effects.

Through the knowledge of both total synthesis and partial modifications of drugs, it has been harnessed to develop better drugs for illnesses and diseases. Both of these techniques were pivotal in our research of finding antidotes against chemical warfare tactics such as nerve agents and even pesticides. Total synthesis and simple modifications of synthetic molecules have paved the way for developing and synthesizing pharmacological drugs against nerve agents. The chemist researching an antidote to nerve agents and pesticides used total synthesis or constructed molecules never made before starting with a simple commercially available starting material.[12,16] The first synthesized organic molecules were to un-inhibit the phosphorylated serine in the acetylcholinesterase's active site. Through retrosynthesis, pyridinium oximes such as 2-PAM was synthesized and successfully reactivate the AChE (Figure 2.5). However, if left untreated after OP inhibition, the OP-inhibited AChE adduct undergoes aging or dealkylation. Currently, there are no known therapeutics to reactivate the aged AChE. The goal of this thesis is to show how the use of synthetic organic chemistry can be used

to construct novel organic molecules and subsequently test them for their ability to reactivate aged AChE.

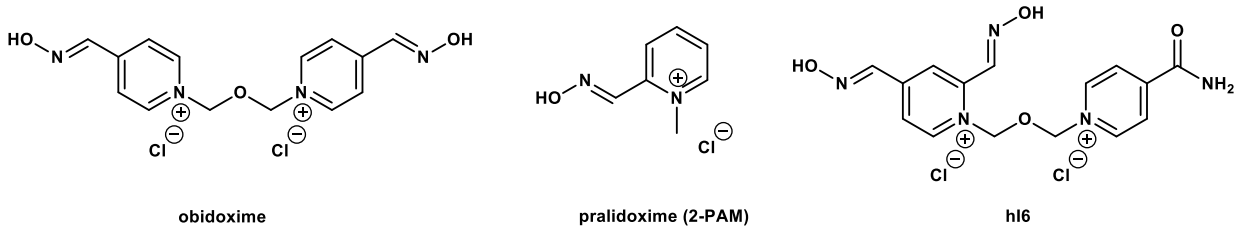


Figure 2.5. Current accepted treatments to reverse OP exposure before enzyme is aged.

The key starting point for our frameworks were derived from the research done by Bakke and Modica's synthesis of quinone methides (QMs).[19] QMs have been used in a biological role of as an alkylator *in vivo*.[19] More specifically, the QMs have alkylated phosphodiester bonds at physiological conditions and proven to reversibly alkylate DNA and block transcription.[19] The modification of the ring and the variability of the amine leaving group can highly dictate the reactive ability of the molecule (Figure 2.6). In addition, the structure of the quinone methide precursors mimic edrophonium, an oxyanilinium-based inhibitor of AChE that is known to bind to native AChE active site.[19] The QM also has the ability to be delivered in an unreactive prodrug form until it is in the AChE active site, where it becomes reactive.[19] Using synthetic synthesis, QMs are derived from benzyl and naphthyl derivatives via silyl cleavage through either oxidative or photochemical means.[19]

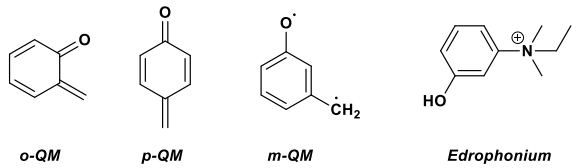


Figure 2.6. General quinone methide structures

Based on the corresponding structures of QMPs and their known characteristics for alkylation and potential affinity for the native AChE active site, our efforts were focused on developing candidates to realkylate aged AChE. More specifically, the *para*-QMPs were synthesized first due to their known affinity for the active site as suggested by computational modeling.[19] In addition to the *para*-QMPs, more modifications were made on the substituents on the ring and the amine leaving group. The library extended to these major five aromatic frameworks (Figure 2.7).

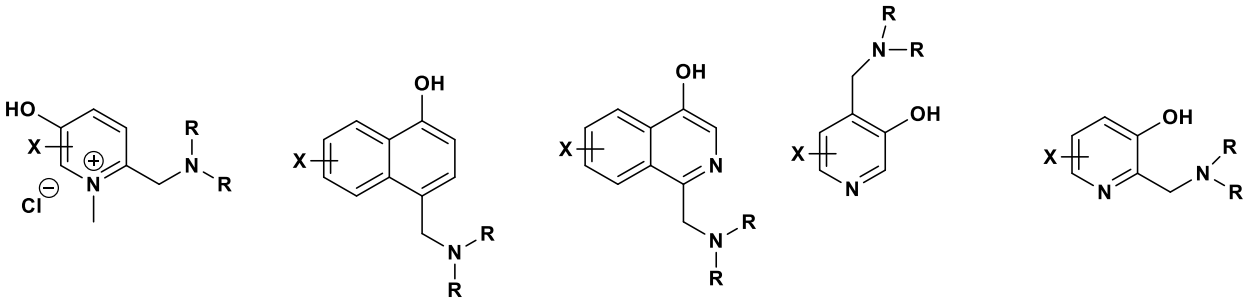
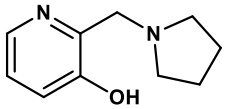


Figure 2.7. Family B: potential aromatic frameworks for quinone methide precursors

Based on family B's success to not only realkylate but also reactivate AChE, we decided to explore other frameworks. After testing this framework, it became the lead compound in resurrecting the aged form of AChE. This lead to my research that probed three major questions. First, how does the amine affect its reactivation capabilities. Secondly, what role does the phenolic group play in the reactivation process. Lastly, how does substitution at the benzylic carbon affect the reactivation capabilities.



2.1

Figure 2.8. One of our most successful resurrectors: 2-(pyrrolidine-1-ylmethly)pyridine-3-ol

Experimental

The methodology of producing re-alkylators and re-activators will be performed with synthesis followed by purification techniques. Synthetic methodology includes setting up a series of chemical reactions in order to make the objective compound. Some type of reactions may include acid/base reactions, nucleophilic substitution, electrophilic substitution, and aromatic chemistry. Through combining these reactions in different orders, the crude products will be produced and then purified.

The goal of purification is to isolate the compound from impurities or by-products produced within the reaction. Some purification techniques that will be performed are crystallization, distillation, gas chromatography, extraction, column chromatography, trituration and filtration. These purifications exploit the differences in the specific characteristics of the organic compounds such as solubility, polarity, and size of the particles. Simple crystallization utilizes the solubilities as a function of temperature. The goal is to have the target compound dissolve in boiling solvent at high temperatures, then as the solution cools down crystals form with only the key compound leaving the impurities in solution.[6] Trituration also takes advantage of the compound's different solubilities. The solvent chosen will dissolve the undesired by-products, leaving the desired product left over.[14] After the recrystallization or trituration, filtration is performed to filter out the impure solution while washing and drying remaining synthetic compound.[7] Distillation is a purification method that separates compounds based on having different boiling points.[18] The more volatile compound will separate and is then cooled to re-liquefy and collect in a container on the other end of the distillation pot or tube. Extraction includes the compounds abilities to dissolve in water or organic solvent by using acid-base reactions.[13]

Compounds can easily be transferred to the ionic form by using an acid or base to either add or eliminate a proton from one of the compounds. The ionic compound will dissolve in the water layer, while the organic compound will remain in the organic solvent, separating out the impurities from the target compound.[13] Column chromatography is a purification method that separates compounds based on their respective polarities.[5] The mobile phase is the substance that moves the solution being purified and stationary-phase interacts with the mobile phase which has different characteristics to hinder certain compounds to elute first.[5]

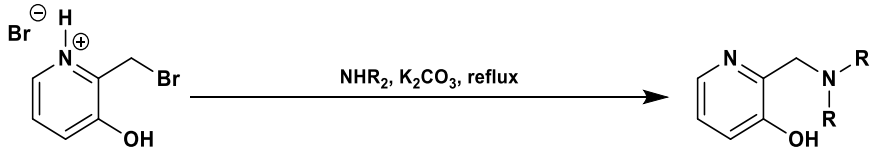
Characterization is determining whether the isolated product is the true synthetic compound that was intended to be made. Specific techniques used will be melting point, boiling point as well as a number of spectroscopic approaches, including nuclear magnetic resonance (^{1}H, ^{13}C and 2D NMR), high resolution mass spectrometry, and infrared spectroscopy. Melting point and boiling point are physical properties that can are used to aid in the identification of the unknown. For melting point, the synthetic compound is believed to be pure if the compound melts in a narrow range of 2 ℃ (narrow range: initial drop appears then when the entire sample dissolves).[2] For boiling point, a pure liquid will distill at constant temperature when pressure above gas the gas is equal to the vapor pressure.[2] The temperatures attained by both, the melting point and boiling point, will be compared to the reported literature range for the pure target compound.[2] ^{1}H-NMR and ^{13}C-NMR methods exploit the behavior of the hydrogens and carbons to determine the carbon-hydrogen framework of the structure of the target organic compounds.[20] Electromagnetic radiation is used to excite or flip the alignment of the nuclear spin to the higher energy opposed state versus the relaxed state spin at lower energy.[20] The larger

the energy difference between these states, then the greater the magnetic field needed to be applied.[20] The position of the peaks on the NMR spectrum depends on the relative electron richness of the H-type or C-type. For ^{13}C-NMR the radio wave pulses coded with angular information determine the number of H's attached to a carbon signal.[20] The chemical shift of the signal produced is based on the chemical groups attached to the carbon-hydrogen bonds. The more electron dense environments, the lower the frequency and the smaller magnetic field applied, the peak is up field. The electron poor environments, the higher the frequency or energy, the larger the magnetic field applied to flip the nuclear spin, the peak is downfield. This equates to the electronegativity of the elements attached. The more electronegative or the more the atom wants to pull the electrons toward itself, then the less electron density shielding the proton or carbon, the greater the peak to the left.[2] Based on these shifts, the chemical compound backbone and structure can be assembled to determine the synthetic compounds purity.[2] Mass spectrometry is used to separate components of a sample by their mass. The sample is ionized and passed through a magnetic field, where a detector can plot the spectrum of different masses using the mass-to-charge ratio. Using this ratio, the molecular formula can be calculated for the compound.[2] Infrared spectroscopy measures the vibration of atoms and based on the vibration produced the functional groups attached.[3]

Construction of the pyridinium molecules: to the effects of steric or Size of the Amine

2.2. 2-Bromomethyl-3-hydroxypyridine hydrobromide framework- S_N2.

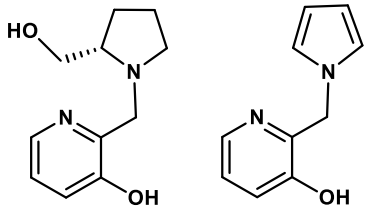
Pyridine QMPs were synthesized from commercially available 2-bromomethyl-3-hydroxypyridine hydrobromide (**2.2**) in a one-step reaction (bimolecular substitution).



2.2

Scheme 2.2. S_N2 reaction conditions for 2-bromomethyl-3-hydroxypyridine hydrobromide frameworks

The experimental steps were the same for each of the reactions except with the change of the secondary amine and temperature of the reactions. This synthesis was used to investigate the space that the amine occupies along with the basicity of the nitrogen. Compound **2.4** has decreased basicity of the nitrogen atom due to it being in an aromatic ring, while Compound **2.3** looks to probe the space and polarity of the hydroxyl methyl group.



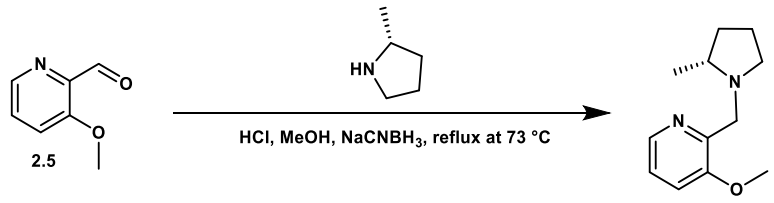
14% yield
2.3

13.9% yield
2.4

Figure 2.9. Target compounds with yields

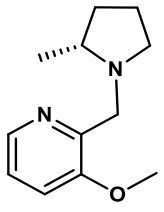
The general experimental procedure for the preparation of these compounds follows with more specific details at the end of the chapter. The reagents of the reaction, 2-bromomethyl-3-hydroxypyridine hydrobromide, amine ((*S*)-2-pyrrolidinemethanol or pyrrole), base (sodium carbonate or sodium hydroxide), and acetonitrile were combined in a round bottom flask and refluxed. The reaction was monitored and removed form heating when the starting material had been consumed. The reaction was monitored by thin layer chromatography. The compound was extracted with organic solution (DCM or diethyl ether), followed by drying and then the solvent was evaporated under reduced pressure. For purification, the compound was analyzed by column chromatography, and then dried and characterized by ^{1}HNMR, ^{13}C NMR, HPLC and mass spectrometry (Appendix A).

The next molecule investigated was methoxy pyridine QMPs. These pyridine QMPs were synthesized from commercially available 3-methoxypicolinaldehyde in a two-step reaction, entailing a reductive amination. The stepwise procedure involved the imine formation followed by the reduction with sodium borohydride, resulting in the QMP.



Scheme 2.3. Reductive Amination reaction conditions for 3-methoxypicolinaldehyde frameworks

This experimental reaction investigates the importance of the phenolic group of the original target compound. The methyl ether group attached to the aromatic pyridine can provide insight into the mechanism of resurrection.



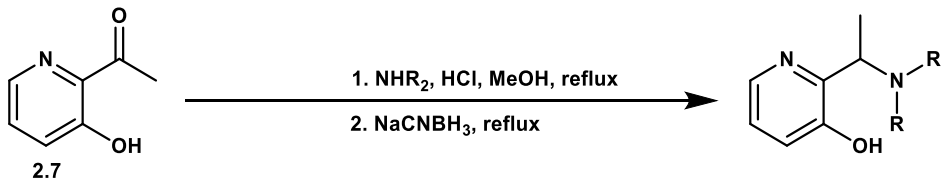
9.20% yield

2.6

Figure 2.10. Target compound with yield

In order to prepare this compound, we started with **2.3** and performed a reductive amination. 3-Methoxypicolinaldehyde was added to an amine, hydrochloric acid, and methanol and stirred at room temperature. The reaction's progress was monitored by TLC until the aldehyde was consumed, followed by addition of the reducing agent (sodium borohydride). The compound was extracted with DCM. After the extraction, the solvent was evaporated under reduced pressure. For purification, the compound was analyzed by column chromatography with ethyl acetate and hexanes to yield the target compound. The compound was dried and characterized by ^{1}HNMR, ^{13}C NMR, HPLC and mass spectrometry (Appendix A).

We also decided to investigate substitution at the benzylic carbon with a methyl group. These compounds were synthesized from the ketone (**2.7**). Pyridine QMPs were synthesized from commercially available 1-(3-hydroxypyridin-2-yl)ethan-1-one (**2.7**) in a two-step reaction, entailing a reductive amination.



2.7

Scheme 2.4. Reductive amination reaction conditions for 1-(3-hydroxypyridin-2-yl)ethan-1-one frameworks

The experimental steps were the same for each of the reactions except with the change of the secondary amine. The addition of the methyl group at the benzylic carbon will provide a better understanding of the mechanism of resurrection with these compounds. The six compounds that were synthesized are shown below.

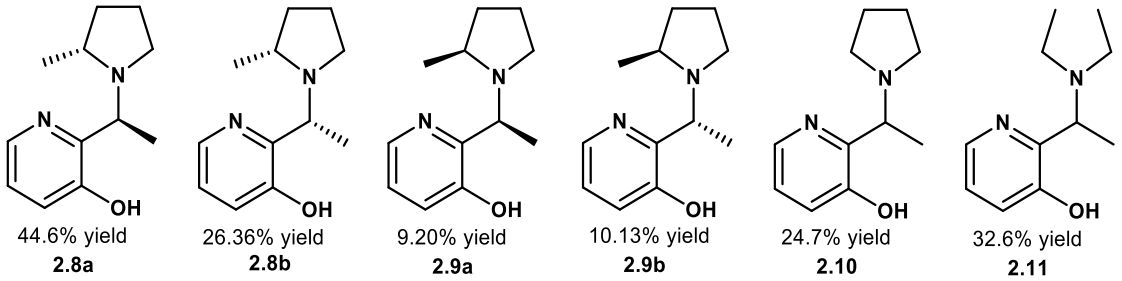


Figure 2.11. Target compounds with yields

The synthesis produced two types of stereoisomers. Compound **2.8** and **2.9** produced a mixture of two diastereomers because a chiral amine was used. For example, the two products produced when (*R*)-2-methyl pyrrolidine was used are the *R*,*R* and *R*,*S* diastereomers as shown in Figure 2.12.

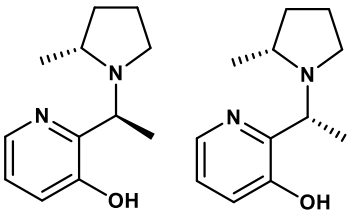


Figure 2.12. 2-(1-((*R*)-2-methylpyrrolidin-1-yl)ethyl)pyridine-3-ol diastereomers

We made both diastereomers but at this time, we do not know the assignment of the configuration at the benzylic carbon after the diastereomers were separated.

The general procedure to produce these compounds is as follows. The amine ((*S*)-2-methylpyrrolidine, (*R*)-2-methylpyrrolidine, pyrrolidine, or diethylamine) was stirred with methanol, while concentrated hydrochloric acid was added dropwise. 1-(3-Hydroxypyridin-2-yl)ethan-1-one was added and stirred at 73 ^{o}C for 12 h to form the imine. The reducing agent was added and refluxed for an additional 12 h at 73 ^{o}C. The solvent was evaporated under reduced pressure.

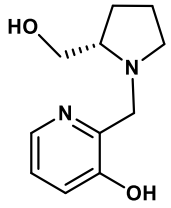
When a chiral amine is used, two diastereomers are made in unequal amounts. When an achiral amine is used, two enantiomers are produced in equal amounts making a racemic mixture. Enantiomers are chiral molecules that are non-superimposable mirror images. Each diastereomer has multiple stereocenters and these compounds are not mirror images and are not superimposable. Enantiomers have identical physical properties except their ability to rotate plane polarized light.[10] Due to enantiomers identical physical properties, in an achiral environment they cannot be separated. Diastereomers have different physical properties and they can be separated.[10] More specifically,

> "one enantiomer may be responsible for the therapeutic effects of a drug whereas the other enantiomer is inactive and/or contributes to undesirable effects. In such a case, use of the single enantiomer would provide a superior medication and may be preferred over the racemic form of the drug. Single-enantiomer formulations of (*S*)-albuterol, a β_2-adrenergic receptor agonist for treatment of asthma, and (*S*)-omeprazole, a proton pump inhibitor for treatment of gastroesophageal reflux, have been shown to be superior to their racemic formulations in clinical trials."[11]

Because AChE is chiral, the diastereomers and enantiomers for compounds **2.8** to **2.11** will have different resurrection reactivities. Therefore, the goal of my purification technique was to separate out the diastereomers to see their individual effects on reactivation of the inhibited AChE. The first attempt to separate the diastereomers included using column chromatography. This technique failed to separate out the two compounds. The next attempt to separate the diastereomers was preparative TLC, which is useful at separating compounds that are close in polarity. This method also failed. The last attempt was high performance liquid chromatography or HPLC.

Using the HPLC techniques, the diastereomers were able to be separated and large enough quantities to be screened. For complete characterization, ^{1}HNMR, ^{13}C NMR, HPLC and mass spectrometry was taken to verify the target compound was synthesized (Appendix A).

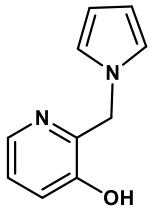
Experimental Details



14% yield

2.3

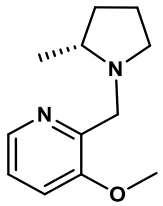
2.3: (*S*)-2-((2-(hydroxymethyl)pyrrolidine-1yl)methyl)pyridin-3-ol. (*S*)-Pyrrolidin-2-ylmethanol (0.0205 mL, 2085 mmol, 2.0 equiv.), acetonitrile (15 mL), 2-bromomethyl-3-hrdroxypyridine hydrobromide (0.280 g, 1.043 mmol, 1.0 equiv.), potassium carbonate (0.288 g, 2.085 mmol, 2.0 equiv.) combined in a round bottom flask and refluxed at 100 °C for 3 h. Distilled water (20 mL) was added to the solution and then was extracted with dichloromethane (4 x 10 mL). The organic layers were collected and dried with anhydrous sodium sulfate, and the solvent was evaporated off under reduced pressure at 35 ºC. The crude product was purified using a column with a gradient of 20%methanol:dichloromethane to yield **2.3** (0.032 g, 0.1450 mmol, 14%).



13.9% yield

2.4

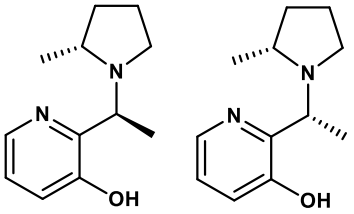
2.4: 2-((1H-pyrrol-1-yl)methyl)pyridin-3-ol. Pyrrole (0.03095 mL, 0.44616 mmol, 1.2 equiv.), acetonitrile (5 mL), 2-bromomethyl-3-hrdroxypyridine hydrobromide (0.100 g, 0.3718 mmol, 1.0 equiv.), potassium hydroxide (0.04589 g, 0.81796 mmol, 2.2 equiv.) combined in a round bottom flask and refluxed at 80 °C for 3 h. Distilled water (10 mL) was added to the solution and then was extracted with diethyl ether (3 x 10 mL). The organic layers were collected and dried with anhydrous sodium sulfate, and the solvent was evaporated off under reduced pressure at 35 ^{o}C. The crude product was purified by column chromatography with a gradient of 20%methanol:dichloromethane to yield **2.4** (0.009 g, 0.0516 mmol, 13.9%).



9.20% yield

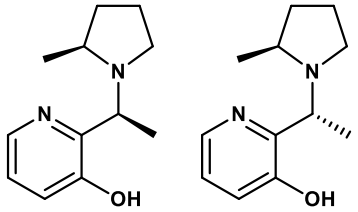
2.6

2.6: (*S*)-3-methoxy-2-((2-methylpyrrolidin-1-yl)methyl)pyridine. (*S*)-2-Methylpyrrolidine (0.044 mL, 0.4375 mmol, 1.5 equiv.) was added to methanol (10 mL) and stirred in a 50mL round bottom flask at room temperature. Hydrochloric acid (0.0155 mL, 0.3208 mmol, 1.72 equiv.) was added dropwise over three minutes to the solution. 3-methoxypicolinaldehyde (0.4 g, 0.2916 mmol, 1 equiv.) was added after the Hydrochloric acid. The solution was refluxed and stirred at 23 °C for 12 h. Sodium cyanoborohydride (0.0504 g, 0.8021 mmol, 1.1 equiv) was added to the mixture after the 12 h reflux. The solution was refluxed and stirred at 73 °C for 12h and then allowed to cool to room temperature. Distilled water (20 mL) was added to the solution and then was extracted with dichloromethane (3 x 10 mL). The organic layers were collected and dried with anhydrous sodium sulfate, and the solvent was evaporated off under reduced pressure at 35 ºC. The crude product was purified by column chromatography with a gradient up to 1:1 hexanes:ethyl actetate to yield **2.6** (0.0111 g, 0.0671 mmol, 9.20%).



44.6% yield **2.8a** 26.36% yield **2.8b**

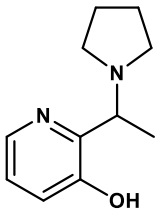
2.8a and 2.8b:2-(1-((*R*)-2-methylpyrrolidin-1-yl)ethyl)pyridine-3-ol. (*R*)-2-Methylpyrrolidine (0.116 mL, 1.09376 mmol, 1.5 equiv.) was added to methanol (10 mL) and stirred in a 50 mL round bottom flask at room temperature. Hydrochloric acid (0.03879 mL, 1.2548 mmol, 1.72 equiv.) was added dropwise over three minutes to the solution. 1-(3-Hydroxypyridin-2-yl)ethan-1-one (1.0 g, 0.7292 mmol, 1 equiv.) was added after the hydrochloric acid. The solution was refluxed and stirred at 73 °C for 12 h. Sodium cyanoborohydride (0.0504 g, 0.8021 mmol, 1.1 equiv.) was added to the mixture after the 12 h reflux. The solution was refluxed and stirred at 73 °C for 12h. The crude product was HPLC to isolate the diastereomers. The solvent was evaporated off under reduced pressure at 75 °C for both diastereomers to yield **2.8a** (0.0673 g, 0.3249 mmol, 44.6%) and **2.8b** (0.0398 g, 0.1921 mmol, 26.36%). Expected Mass [$C_{12}H_8N_2O$ = 206.1419], [M+H] **2.8a.** 207.1551; [M+H] **2.8b.** 207.1549



9.20% yield
2.9a

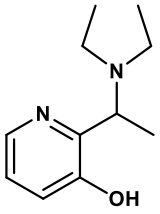
10.13% yield
2.9b

2.9a and 2.9b: 2-(1-((*S*)-2-methylpyrrolidin-1-yl)ethyl)pyridine-3-ol. (*S*)-2-Methylpyrrolidine (0.116 mL, 1.09376 mmol, 1.5 equiv.) was added to methanol (10 mL) and stirred in a 50mL round bottom flask at room temperature. Hydrochloric acid (0.03874 mL, 1.2542 mmol, 1.72 equiv.) was added dropwise over three minutes to the solution. 1-(3-Hydroxypyridin-2-yl)ethan-1-one (1.0 g, 0.7292 mmol, 1 equiv.) was added after the hydrochloric acid. The solution was refluxed and stirred at 73 °C for 12 h. Sodium cyanoborohydride (0.0504 g, 0.8021 mmol, 1.1 equiv.) was added to the mixture after the 12 h reflux. The solution was refluxed and stirred at 73 °C for 12 h. The crude product was HPLC to isolate the diastereomers. The solvent was evaporated off under reduced pressure at 75 ºC for both diastereomers to yield **2.9a** (0.0139 g, 0.0671 mmol, 9.20%) and **2.9b.** (0.0153 g, 0.07386 mmol, 10.13%). Expected Mass [$C_{12}H_8N_2O$ = 206.1419], [M+H] **2.9a.** 207.1556; [M+H] **2.9b.** 204.1553



24.7% yield
2.10

2.10: 2-(1-(pyrrolidin-1-yl)ethyl)pyridine-3-ol. Pyrrolidine (0.0913 mL, 1.09376 mmol,1.5 equiv.) was added to methanol (10 mL) and stirred in a 50 mL round bottom flask at room temperature. Hydrochloric acid (0.03879 mL, 1.2548 mmol, 1.72 equiv.) was added dropwise over three minutes to the solution. 1-(3-Hydroxypyridin-2-yl)ethan-1-one (1.0 g, 0.7292 mmol, 1 equiv.) was added after the Hydrochloric acid. The solution was refluxed and stirred at 73 °C for 12 h. Sodium cyanoborohydride (0.0504 g,0.8021 mmol, 1.1 equiv.) was added to the mixture after the 12 h reflux. The solution was refluxed and stirred at 73 °C for 12 h. The crude product was purified by HPLC to isolate the diastereomers. The solvent was evaporated off under reduced pressure at 75 °C to yield **2.10** (0.0348 g, 0.1801 mmol, 24.7%). Expected Mass [$C_{11}H_{16}N_2O$ = 192.1263], [M+H] 193.158



32.6% yield
2.11

2.11: 2-(1-(diethylamio)ethyl)pyridine-3-ol. Diethylamine (0.06006 mL, 0.80212 mmol, 2.2 equiv.) was added to methanol (10 mL) and stirred in a 50mL round bottom flask at room temperature. Hydrochloric acid (0.01973 mL, 0.62709 mmol, 1.72 equiv.) was added dropwise over three minutes to the solution. 1-(3-h]Hydroxypyridin-2-yl)ethan-1-one (0.5 g , 0.3546 mmol, 1 equiv.) was added after the hydrochloric acid. The solution was refluxed and stirred at 73 °C for 12 h. Sodium cyanoborohydride (0.0252 g, 0.4010 mmol, 1.1 equiv.) was added to the mixture after the 12 h reflux. The solution was refluxed and stirred at 73 °C for 12 h. The crude product was purified by HPLC to isolate the diastereomers. The solvent was evaporated off under reduced pressure at 75 °C to yield **2.11** (0.0225 g, 0.1158 mmol, 32.6%). Expected Mass [$C_{11}H_{18}N_2O$ = 194.1419], [M+H]:194.270

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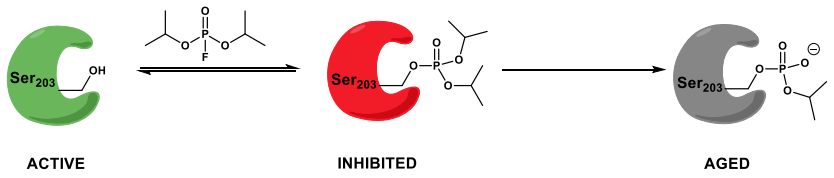
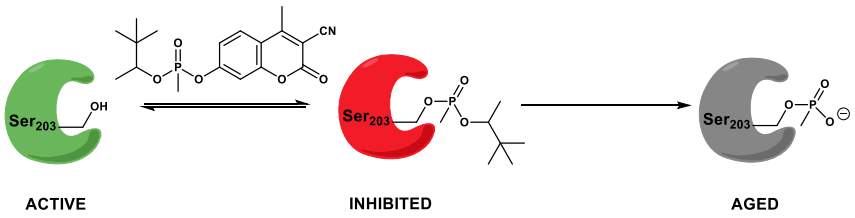
Chapter 3: Biological Testing and Future Work

3.1 Kinetic Studies

Once the compounds were purified and characterized, they are tested for the alkylator's biological effects with the aged form of AChE. Initially, electric eel AChE was added with an OP. However, the positive control was native AChE that had not been exposed to an OP (native AChE). The negative control was the aged AChE; however, no QMPs, purified compounds, or 2-PAM were added. The positive and negative controls are important for comparing the evaluated compound's biological effect and its significance in resurrecting some activity of the native AChE. The negative control is only aged AChE with no addition of any compound; the aged AChE activity of the negative control provides a baseline for the small percentage that did not fully age. The amount of unaged AChE is usually less than 0.5% for the negative control. This percentage is compared to the percentage of resurrected AChE when the newly synthesized molecules are added to ensure the percentage is higher than the negative's control. If the percentage is close to zero or the negative control's percentage, then the tested molecule is inactive and has no effect on the realkylating or resurrecting of the aged form of AChE. For testing the purified compounds described in Chapter 2, positive and negative control for two different OPs, specifcially PiMP and DFP, were used as the AChE inhibitors (Scheme 3.1 and 3.2).

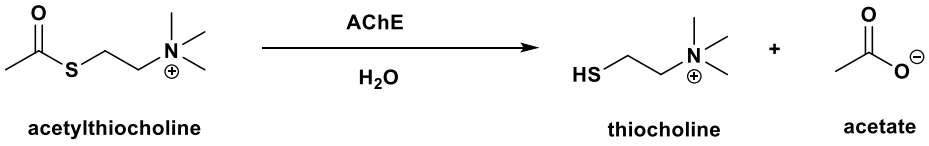


Scheme 3.1. Aging of AChE with DFP

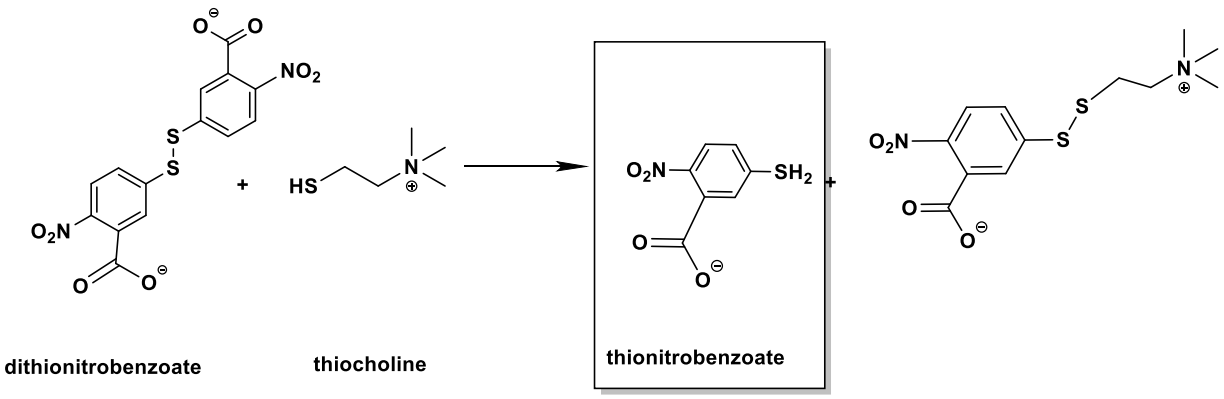


Scheme 3.2. Aging of AChE with PiMP

PiMP is a simulant of an authentic chemical warfare agent or nerve agent while DFP is a pesticide. Ellman's assay is used to determine the activity of acetylcholinesterase (AChE). Ellman's assay monitors the activity of the enzyme by hydrolyzing acetylthiocholine into thiocholine instead of acetylcholine to choline (Scheme 3.3).[1] Then, the thiocholine will then reduce the disulfide bonds in dithionitrobenzoate to thionitrobenzoate plus another by-product (Scheme 3.4).[1]



Scheme 3.3. Hydrolysis of acetylthiocholine



Scheme 3.4. The reaction of dithionitrobenzoate and thiocholine in an Ellman's assay

Therefore, activity of the enzyme is monitored by the concentration of thionitrobenzoate via ultraviolet-visible spectroscopy because thionitrobenzoate absorbs light at 412 nm.[1] Therefore, the absorbance directly correlates with more reactivated AChE. The alkylators and pure synthetic compound will be incubated with aged electric eel AChE for 2 days at 37 °C.[1] The pure synthetic compound should re-alkylate the inhibited AChE like a pyridinium oxime.[1] Based on the results of this last step, the target alkylator being evaluated will be modified for the next synthetic molecule and retested if it does not properly realkylate and reactivate the AChE.

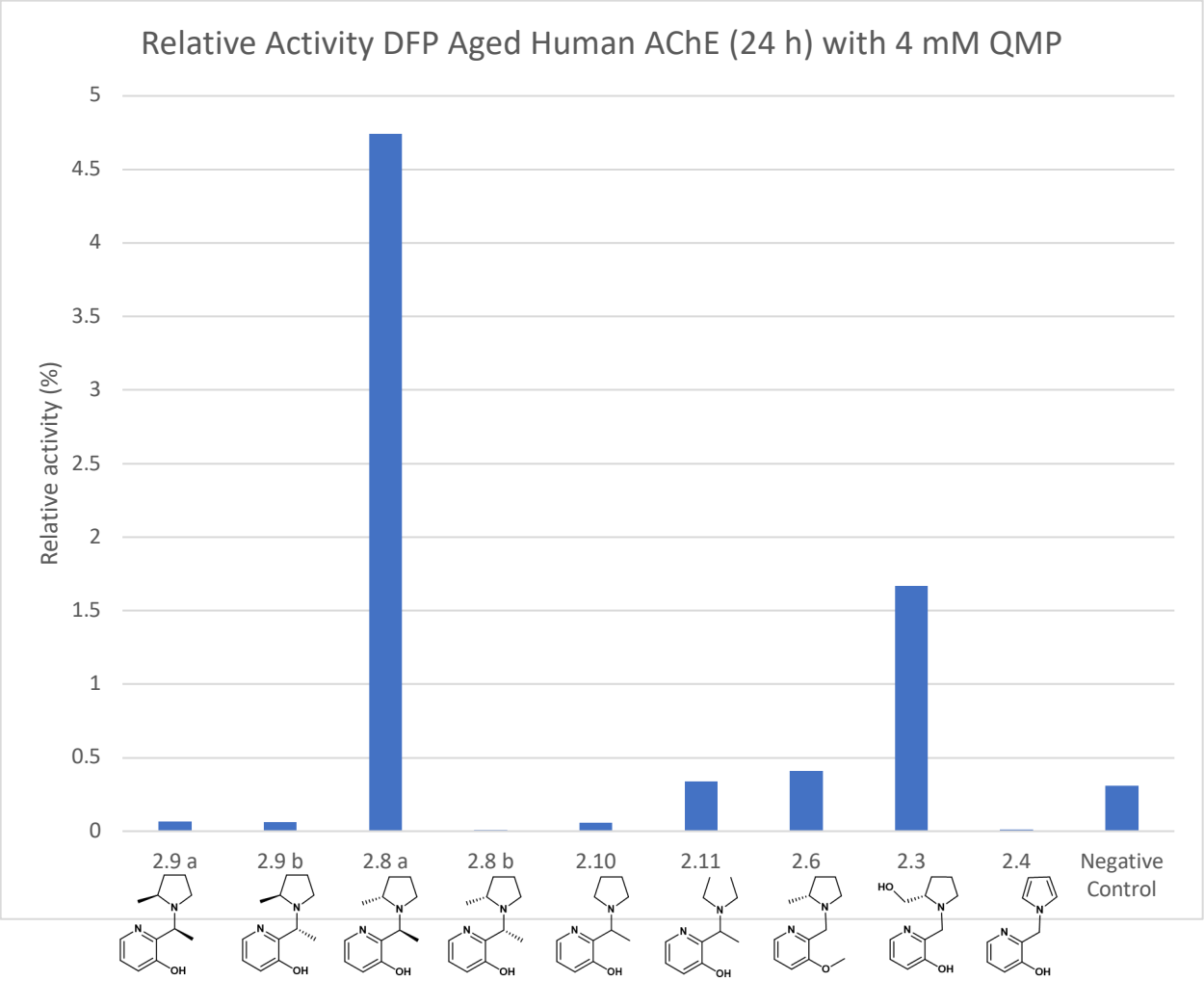


Figure 3.1. Results from the screening

Although the goal is to have the highest relative reactivation, it has been proven by many sources that 10% reactivation of AChE is needed for human function. This percentage will be compared to the results shown in the screening. The results of the screening of the compounds presented in this thesis are shown in Figure 3.1. The positive control is not shown in the figure for simplification of presentation, but all values are relative to the positive control at 100%. Compound **2.9 a**, **2.9 b**, **2.10**, **2.11**, **2.6** and **2.3** showed no activity. The activity AChE in the samples was less than or equal to the negative control. Compounds **2.8 a** and **2.3** show activity in the screen's assay or the bars were higher than the negative control by +/- 1%. More specifically, the results will be

broken down and compared to the lead compounds discussed earlier in the thesis. The percentage displayed for the lead compounds (**2.1** and **2.12**) were all at the same pH of 7.5, aged DFP and Human AChE. The same condition that the tested compounds were done in.

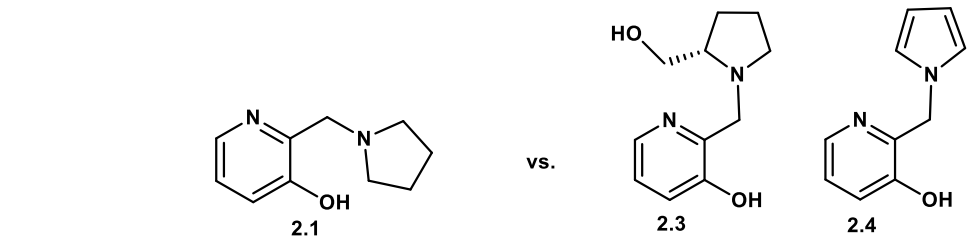


Figure 3.2. Relative resurrection between compounds **2.1**, **2.3**, and **2.4**

As seen in Figure 3.2, compound **2.3** is very similar to the lead compound discussed earlier in this thesis. The presence of the hydroxymethyl group added on the pyrrolidine decreases activity but it seems that the active site within the enzyme tolerates the presence of that group, but with decreased selectivity. The low activity of compounds **2.4** indicate that the basicity of the nitrogen plays an important role in the resurrection.

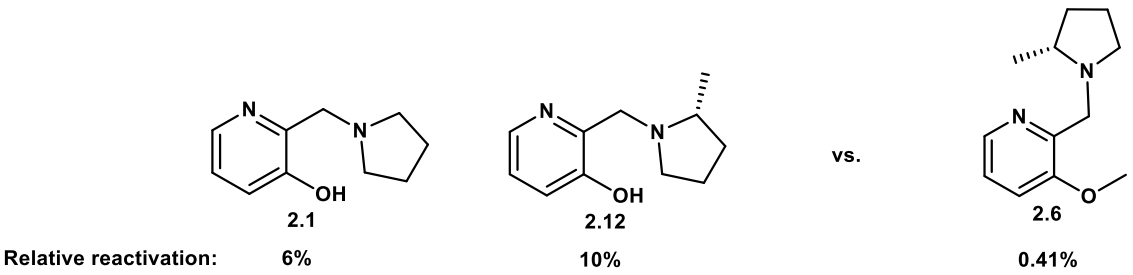


Figure 3.3. Relative resurrection between compounds **2.1**, **2.12**, and **2.6**

Figure 3.3 shows a comparison of the relative resurrection of the lead compound to **2.6**. The major difference between **2.6** compared to **2.1** and **2.12** is the ester group instead of the hydroxyl group on the ring. The low activity of compound **2.6** demonstrates the presence of the phenolic group play an important role in the resurrection ability of our compounds.

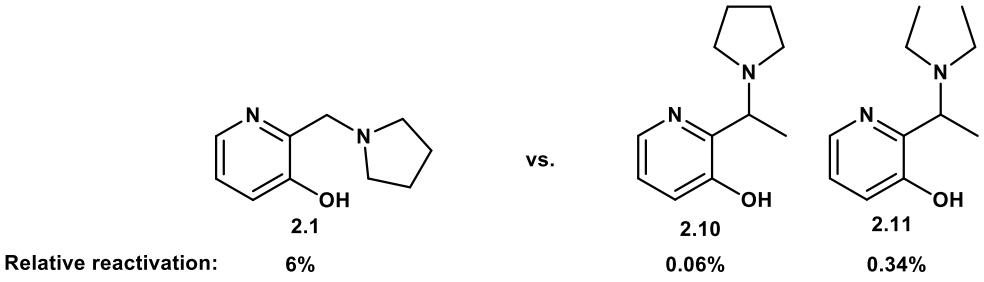


Figure 3.4. Relative resurrection between **2.1**, **2.10** and **2.11**

Figure 3.4 shows a comparison of the relative activator of the lead compound **2.1** to **2.10** and **2.11**. The low activity of compound **2.10** demonstrates that adding a methyl group at the benzylic carbon as a negative effect the resurrection of the aged AChE. Similarly, the low activity of compound **2.11** shows the addition of methyl group regardless of amine reduced resurrection of the aged AChE was observed. However, as stated above, **2.10** and **2.11** are racemic. The different enantiomers can play different

roles in the active site. Therefore, the low activity could be due to one enantiomer being an inhibitor to the enzyme.

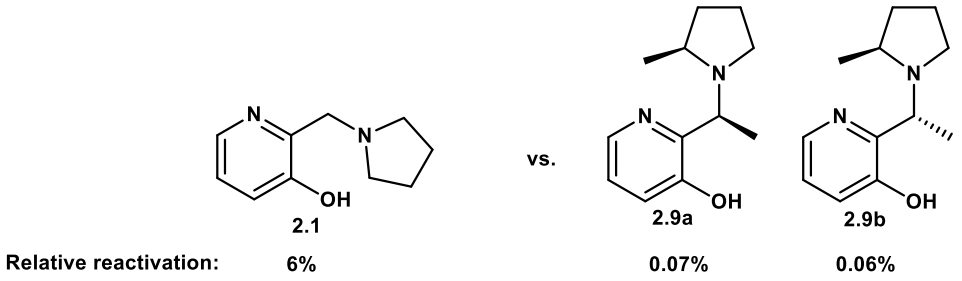


Figure 3.5. Relative resurrection between **2.1**, **2.9 a** and **2.9 b**

Figure 3.5 shows a comparison of the relative resurrection for **2.1**, **2.9 a**, and **2.9 b**. The low activity of **2.9 a** and **2.9 b** indicate the addition of the methyl group at the benzylic carbon and (S)-methylpyrrolidine had a negative effect on the resurrection of the aged AChE.

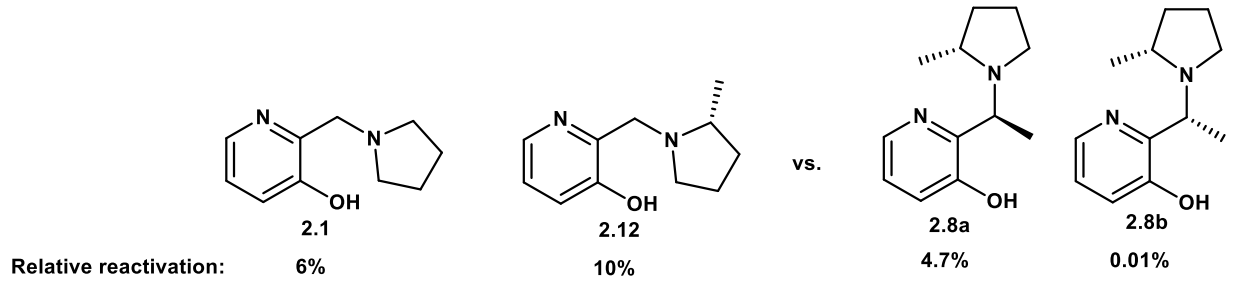


Figure 3.6. Relative resurrection between **2.1**, **2.12**, **2.8a**, and **2.8b**

Figure 3.6 shows a comparison of both the compound's structures and relative resurrection for **2.1**, **2.12**, **2.9 a**, and **2.9 b**. The most interesting values are the data for the diastereomeric set **2.8 a** and **2.8 b**. This set of diastereomers were not able to be completely separated when HPLC isolation was attempted. Therefore, the screening results presented above show the activity as a mixture of two diastereomers for entry **2.8**

a and **2.8 b**. For entry **2.8 a** the less polar isomer is in excess by 10% and for entry **2.8 b** the more polar isomer by HPLC is in excess by ~11%. It is remarkable that with only a slight excess in one of the diastereomers, the sample screened for **2.8 a** showed a 4.7% resurrection while when the other diastereomers is in excess in **2.8 b**, the sample shows no activity. This could indicate that one of the diastereomers is a potent resurrector while the other compound is a potent inhibitor of the enzyme. Compound **2.9a** and **2.9b** did not show any activity indicating the stereochemistry of the 2-methylpyrrolidine needs to be the *R* configuration for activity. This result does not only prove chirality is significant in resurrection but also the addition of a substituent to the benzylic carbon also plays a key role. The addition of the methyl group to the benzylic carbon may also play a key role in resurrection because this compound may have more favorable conditions to form the QM resulting in this high relative activity. Our immediate task at hand is to resynthesize these frameworks and to improve the separation by HPLC so that we can confirm this idea and further probe the resurrection capabilities of this framework when in their pure form.

Due to the advancements in chemical warfare agents more specifically the toxic OPs that have devasting effects on individuals leading a demand for find counter measures. Although there are drugs that reactivate inhibited AChE, these current treatments have limitations. The drugs are positively charged; therefore, they can not pass the blood brain barrier can lead to a build up ACh in the brain causing fatal effects. In addition, if the drugs are not dosed correctly or administered in the correct time frame the inhibited AChE will become irreversibly dealkylated or aged. There are currently no known treatments that for re-alkylation of the aged AChE to then administer the current drugs to reactivate the aged AChE or resurrects the aged AChE. (Figure 3.7) The goal of

my research is to synthesize a small organic molecule that will resurrect the aged AChE. Based on my results, Compound **2.8 a** showed promising results to further research and optimize its resurrection capabilities.

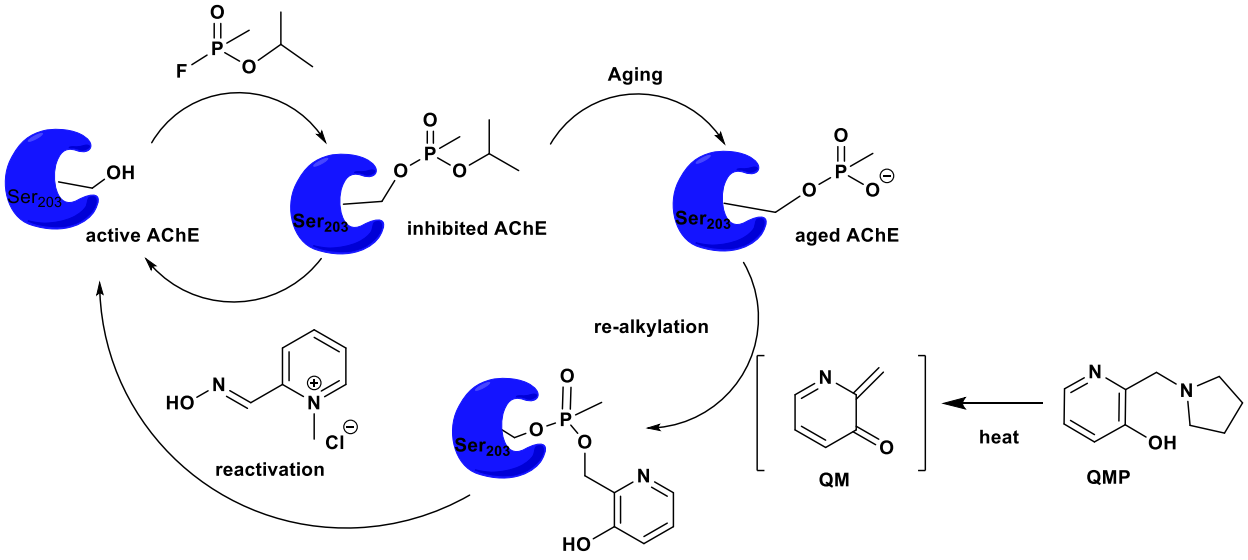
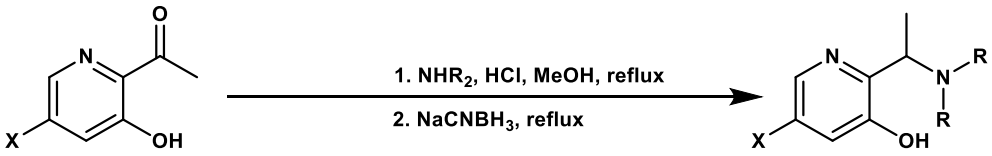


Figure 3.7. Research significance

3.2 Future work

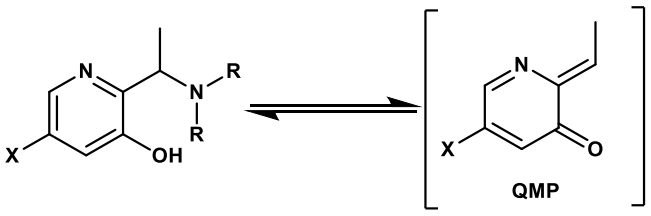
Based on the results from the kinetic study shown above, the molecules can be adapted to further increase its reactivity results. The diastereomers from the (*R*)-2-methylpyrrolidine showed the highest reactivity. Using this framework as the goal, we plan to add other substituents to the pyridine ring to see the effects on the compounds rate of forming the QMP or its percent reactivation (Scheme 3.5).



X= Electron dontating group (EDG) or Electron withdrawing group (EWG)

Scheme 3.5. Ethanone reduction with the addition of EDG or EWG

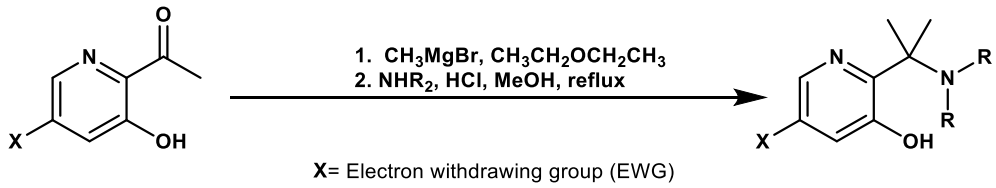
The added substituent, X, can either be an electron donating group or electron withdrawing group. Based on the characteristic of an electron donating group, it is hypothesized that the electron donating group would increase the rate of formation of the QMP compared to the electron with drawing group (Scheme 3.6). This is because the electron donating group stabilizes the carbocation that forms making the QMP formation more favorable. In comparison, the electron withdrawing group destabilizes the carbocation making the QMP less likely to form.



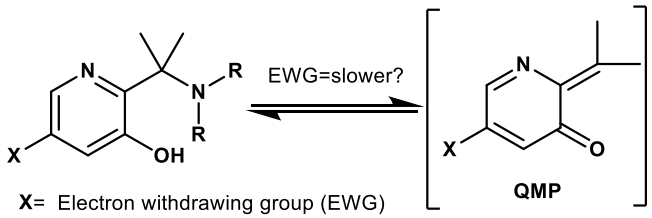
X= Electron dontating group (EDG) or Electron withdrawing group (EWG)

Scheme 3.6. QMP equilibrium

In addition to adding either an electron withdrawing or electron donating group, another question is adding another methyl group to the benzylic carbon (Scheme 3.7). The addition of another methyl group previously has been synthesized; however, the targets compound is very unstable and degrades spontaneously in water, relative to the QMP that has only one methyl group at this position. This is because the carbocation formed is a tertiary center with more substituents to stabilize the charge compared to the secondary carbon with one methyl group at the benzylic center. Therefore, the goal is to combine the addition of the methyl group at the benzylic carbon and an EWG to the pyridine ring to try and stabilize the target compound (Scheme 3.8). The EWG should make the benzylic carbon less favorable to form the carbocation since it will withdraw electrons from the tertiary center.



Scheme 3.7. Ethanone reduction with the addition of EDG or EWG and methyl group



Scheme 3.8. QMP equilibrium

Work Cited

1. Fabry, S. Synthesis of Flurobenzyl Alkylators : Studies Toward Realkylation. *Thesis, Ohio State Univ.* , **2016**.

Appendix A

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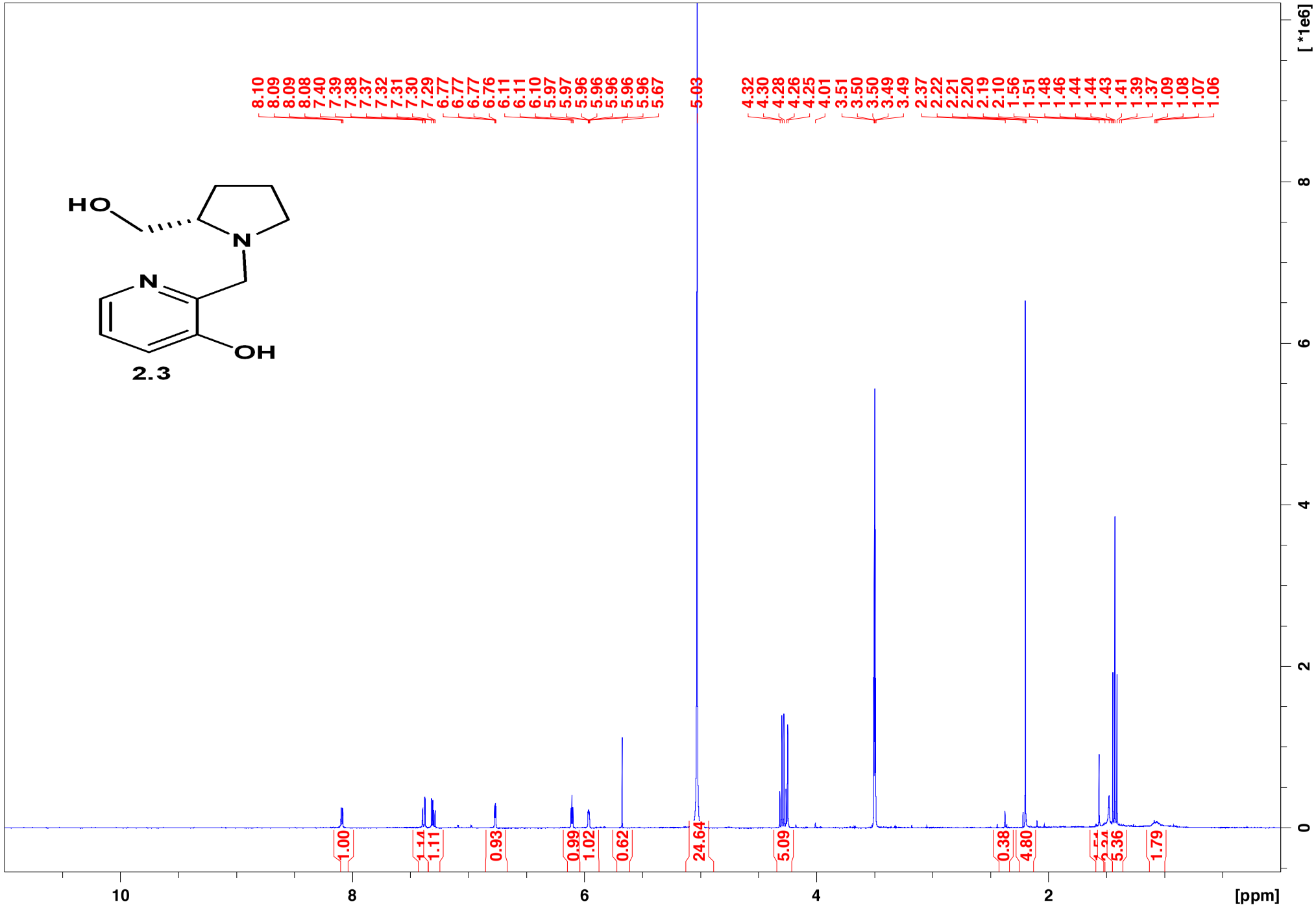

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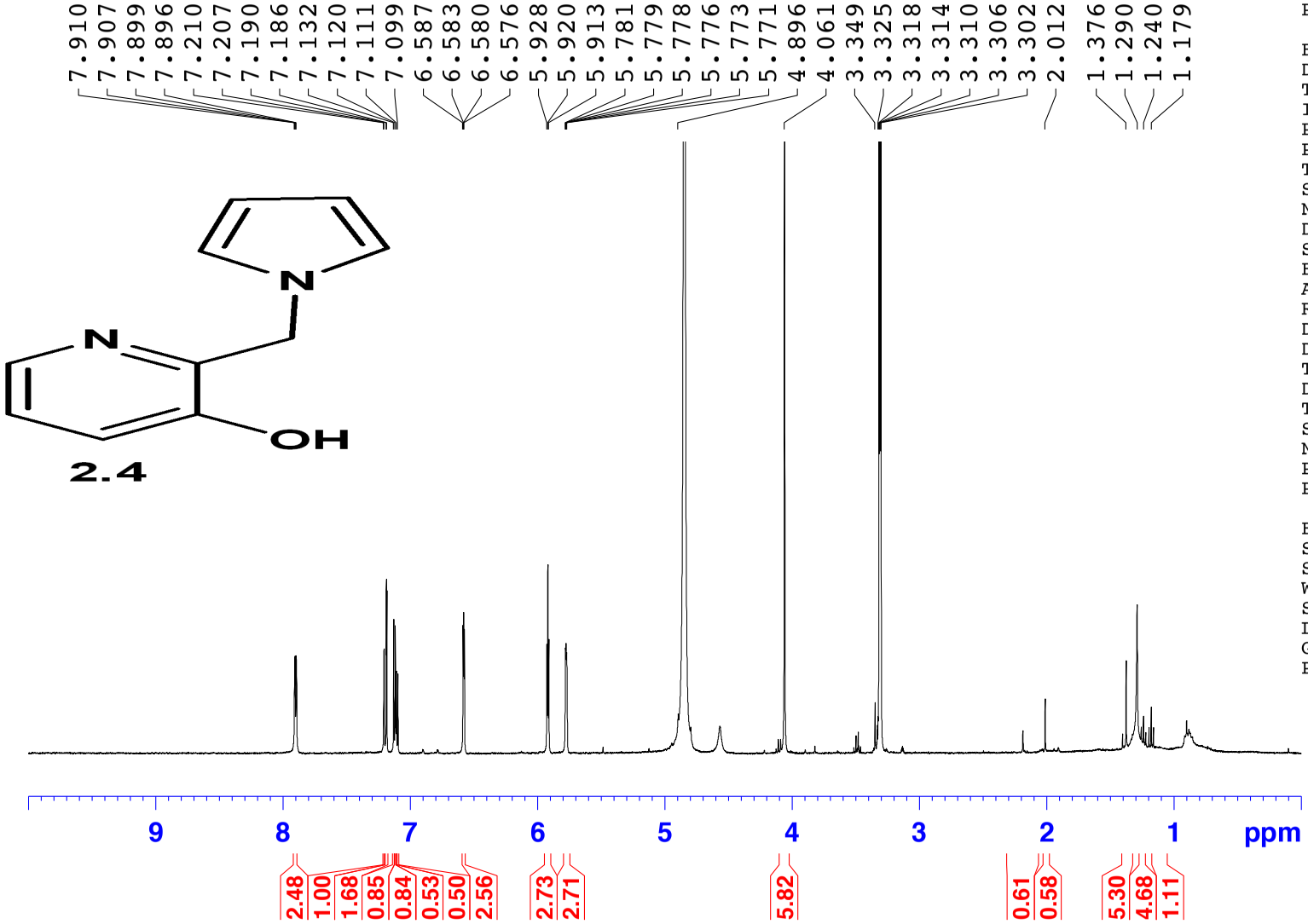

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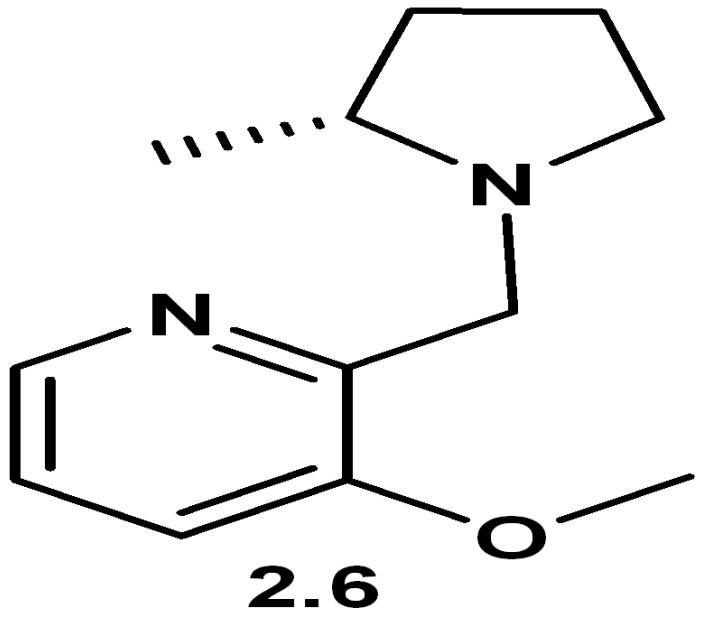

2.6

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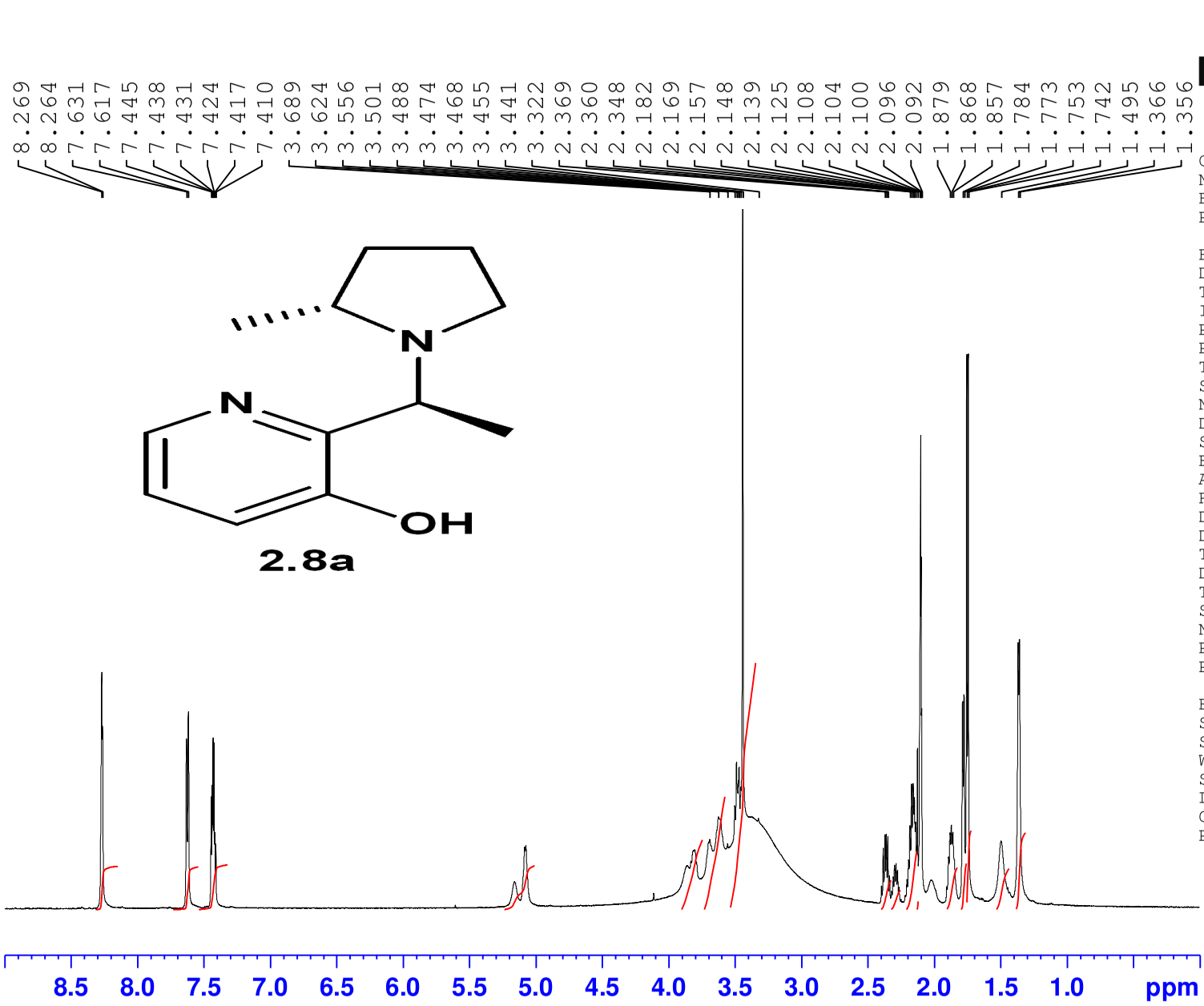

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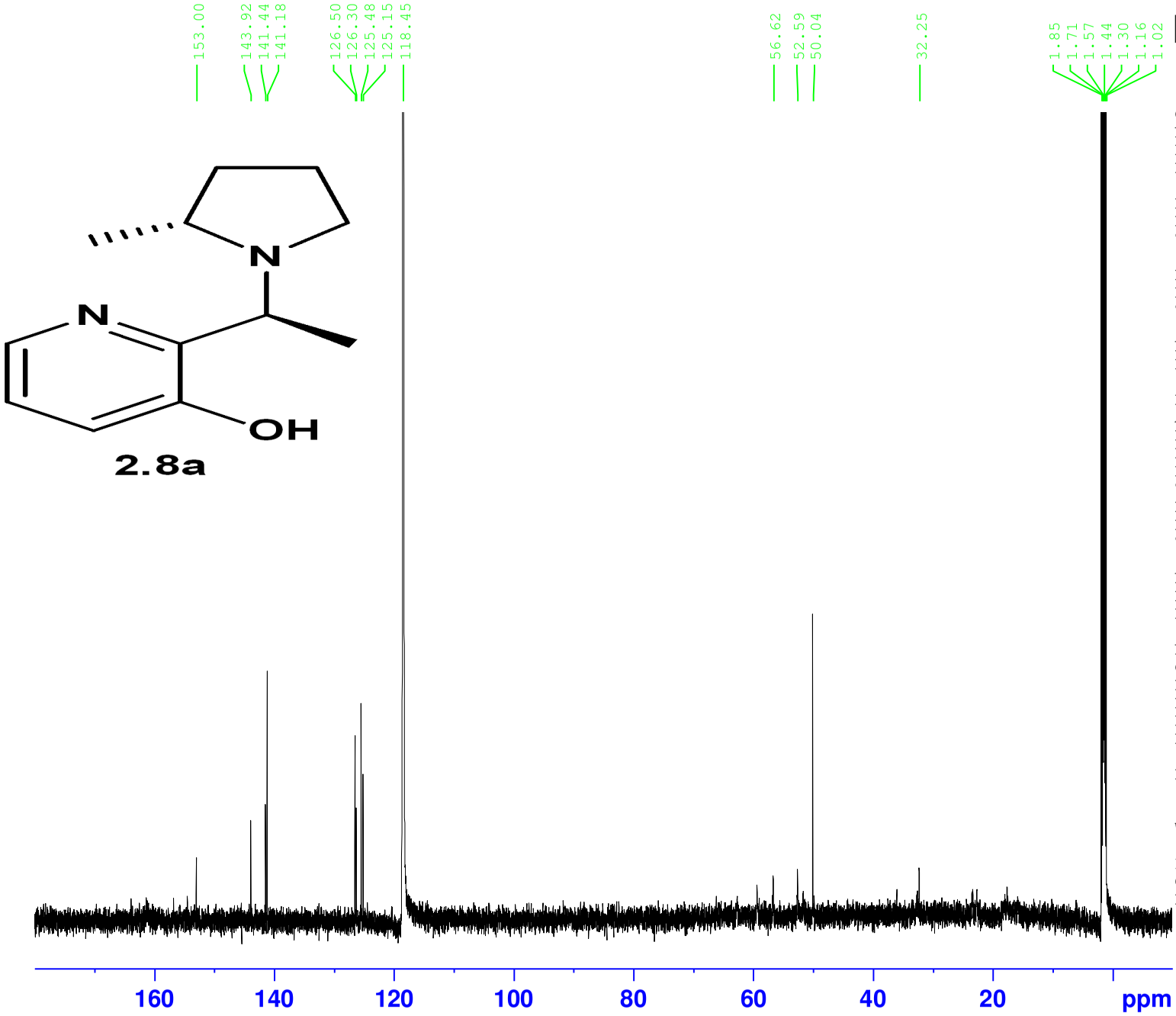

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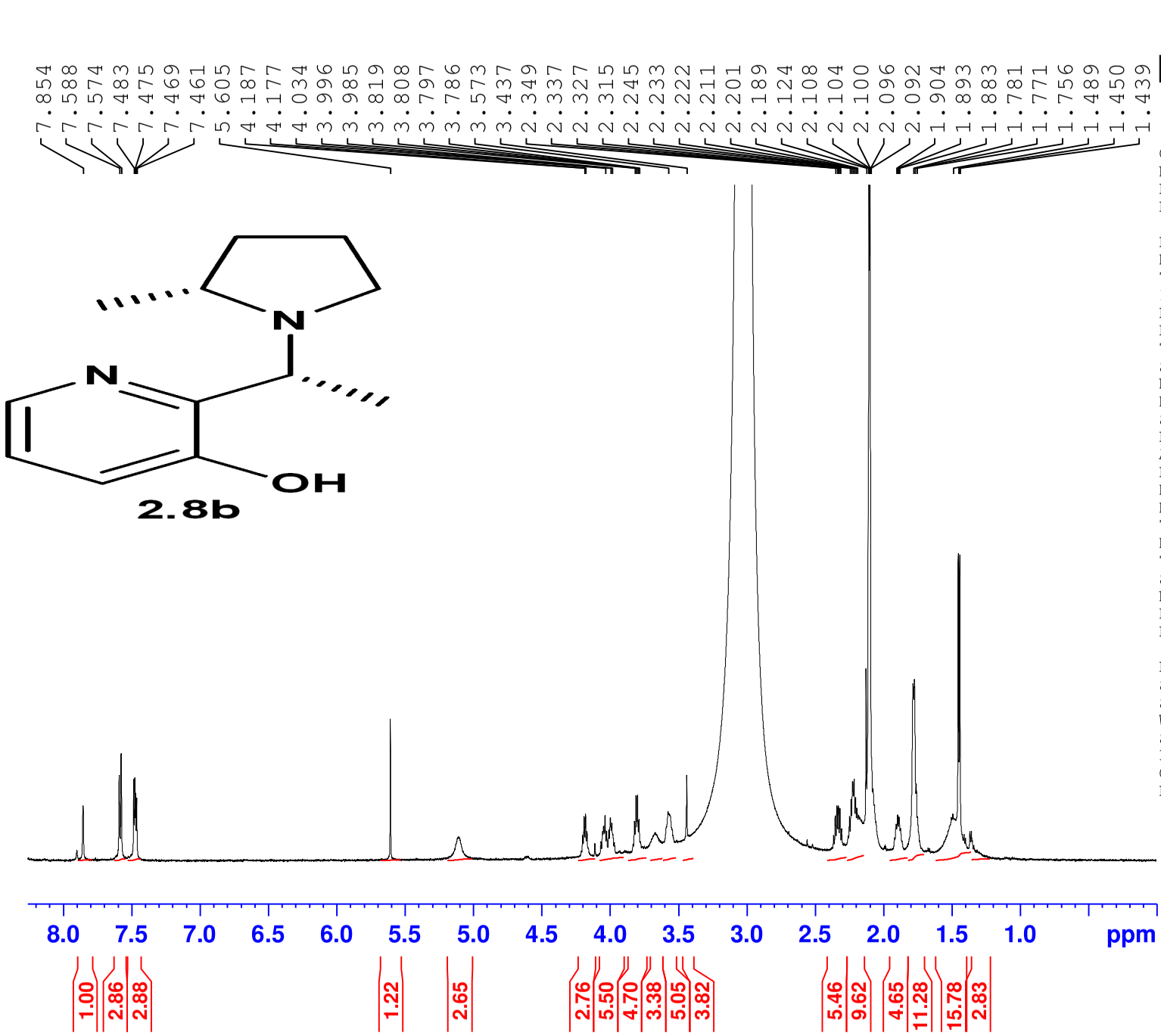

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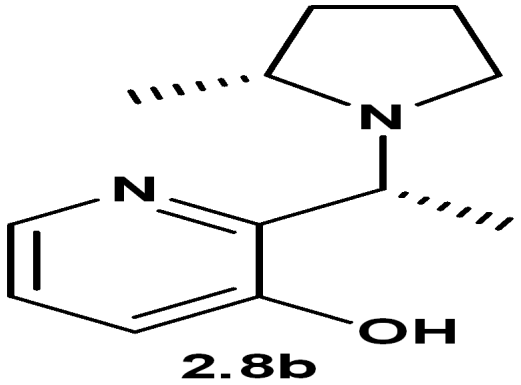

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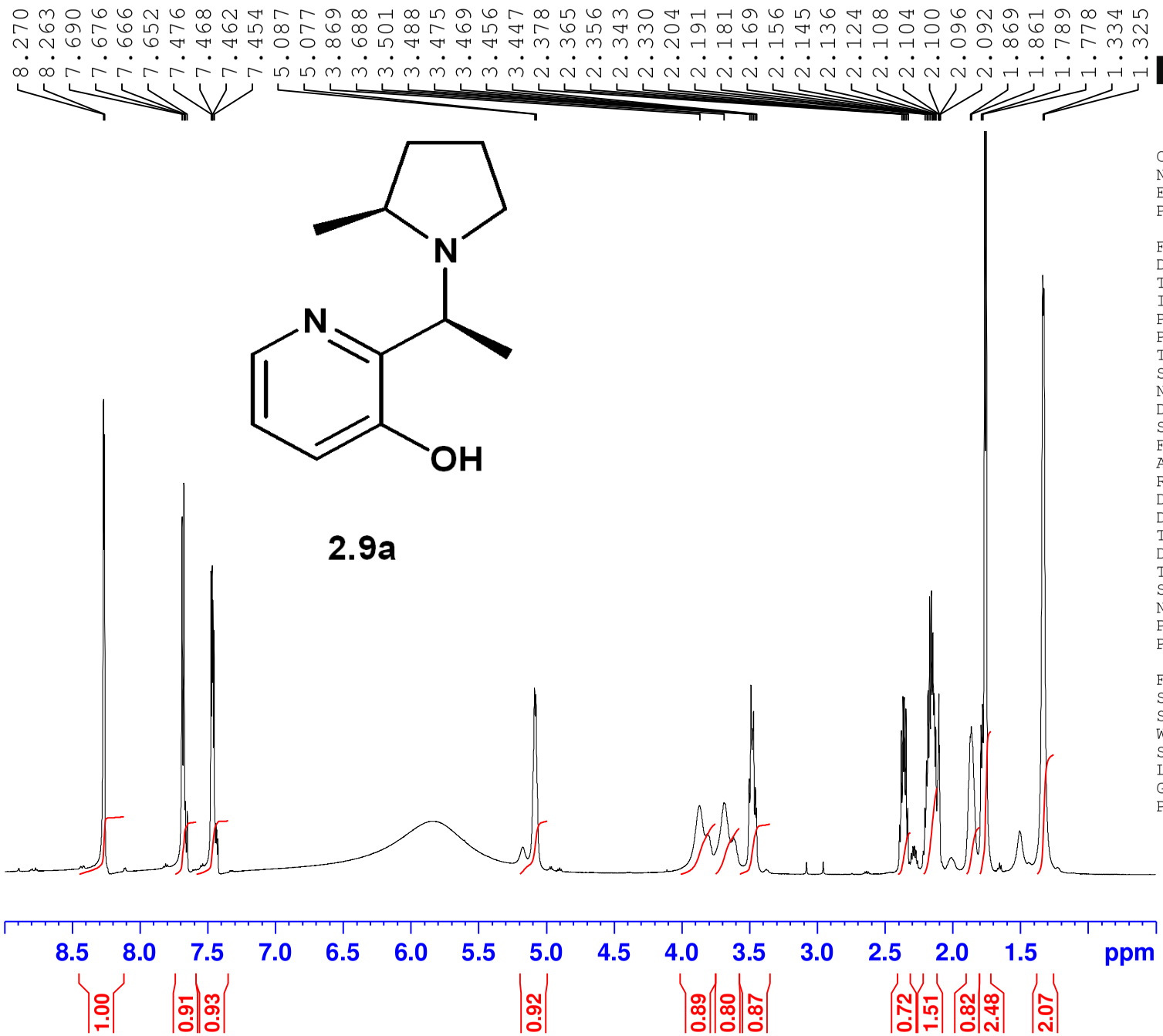


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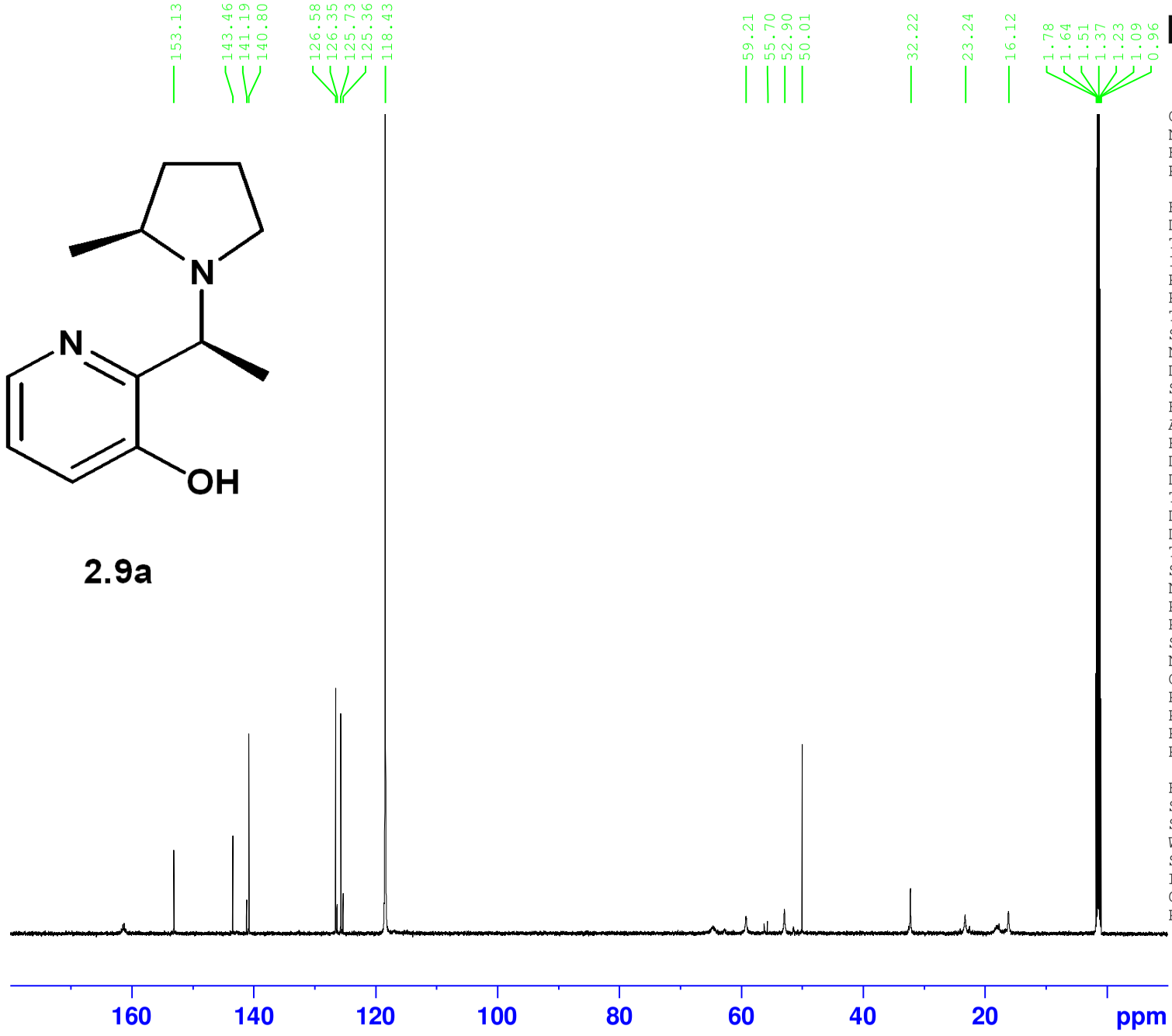

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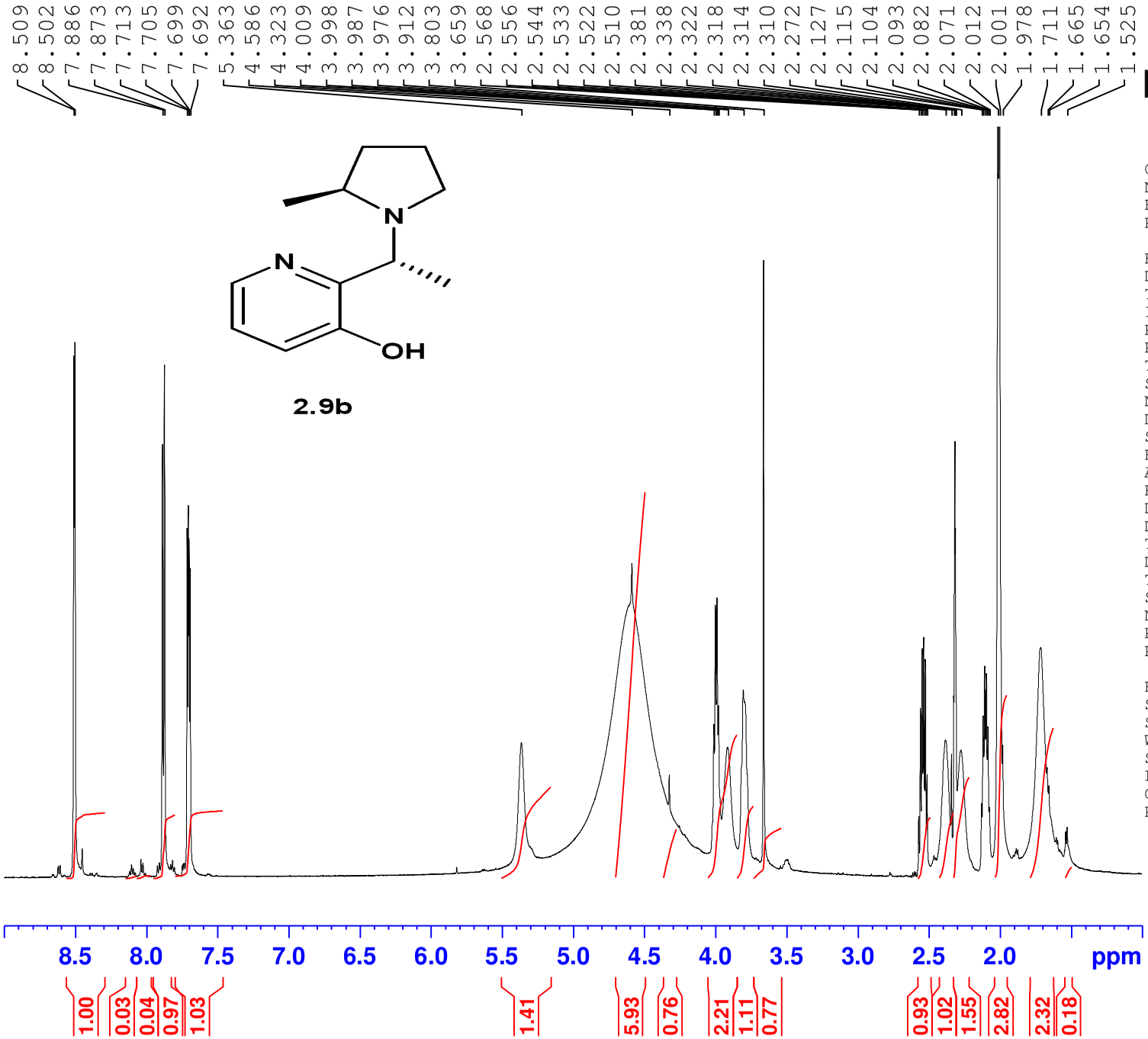

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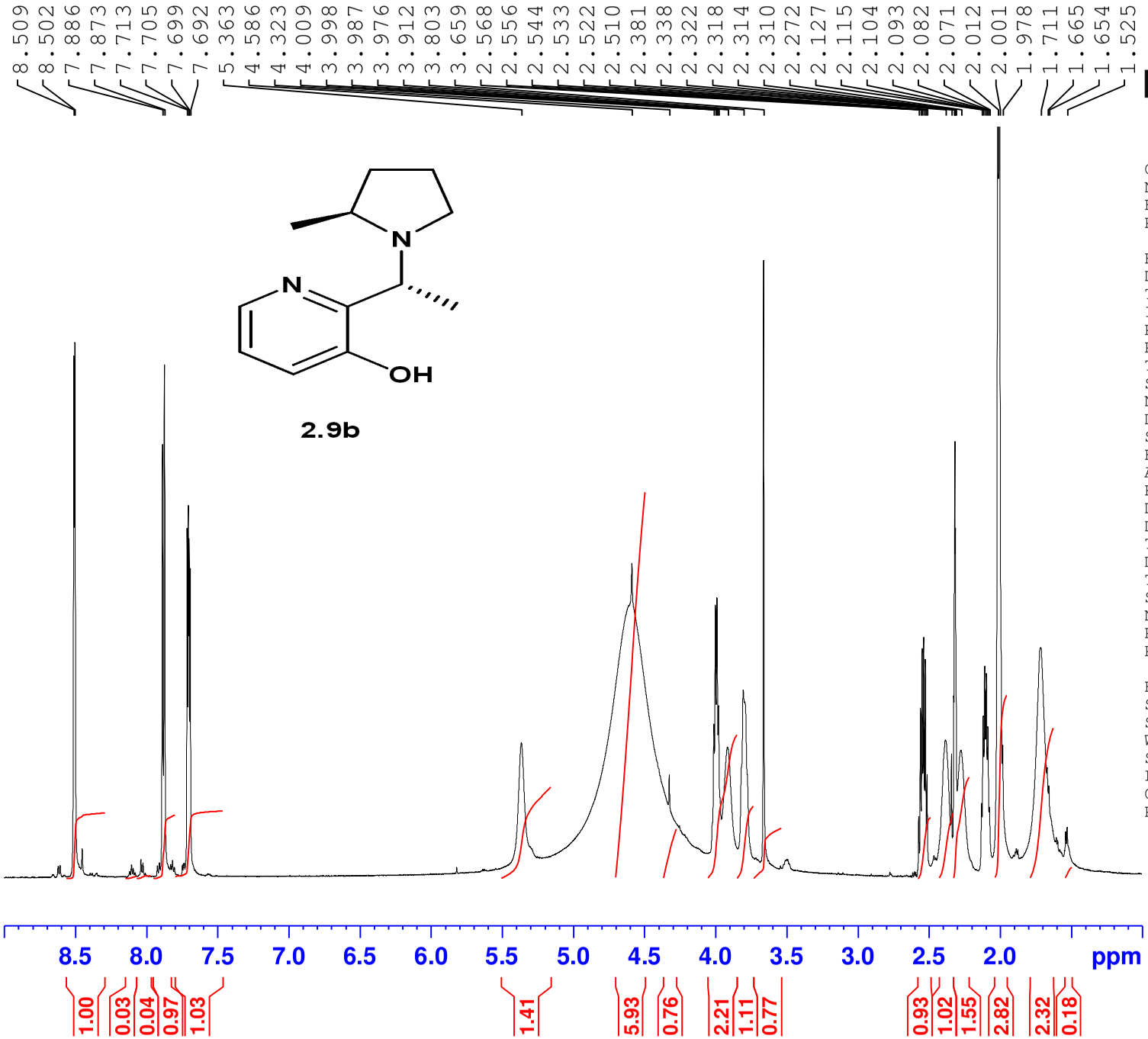

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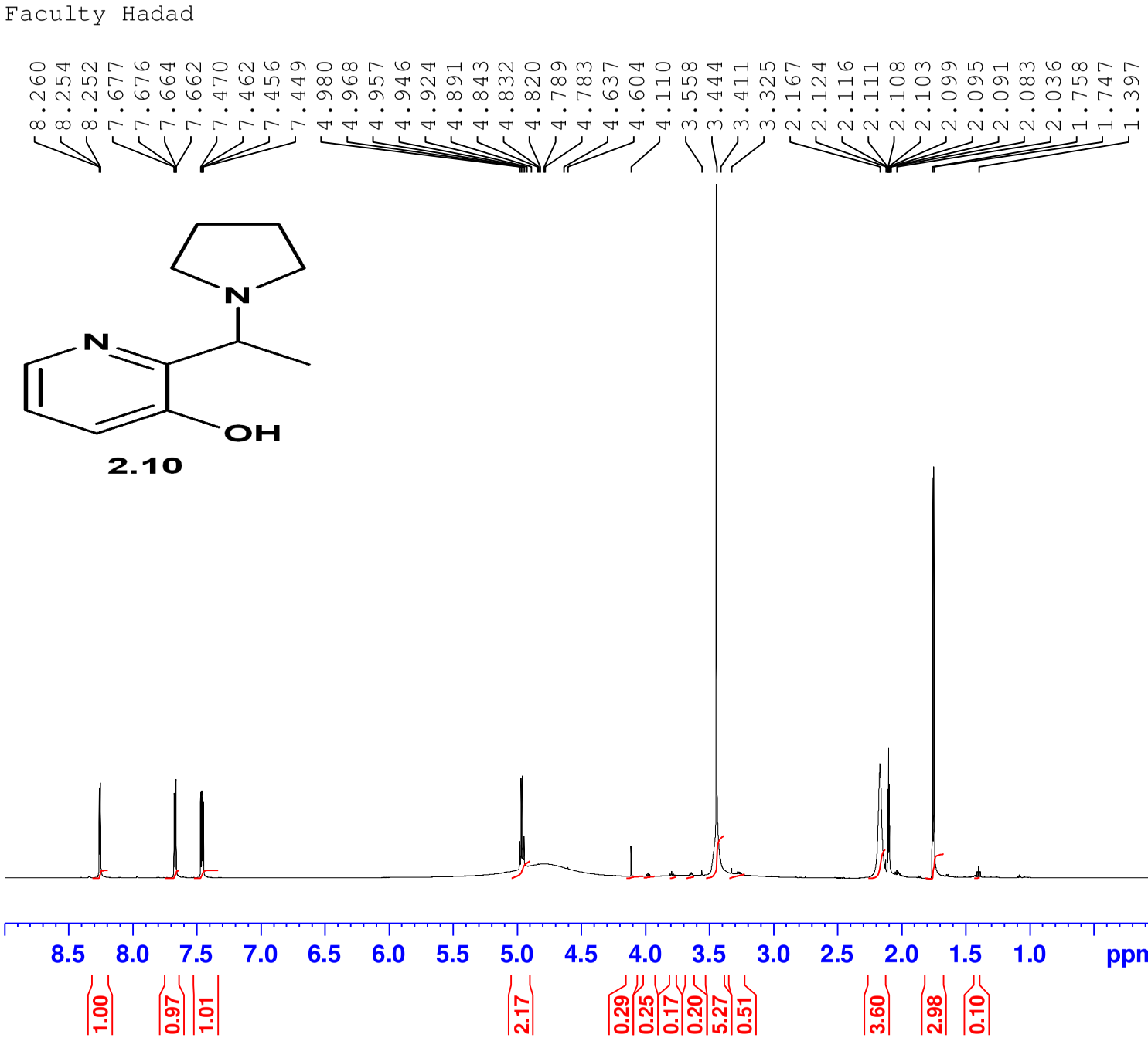
Faculty Hadad



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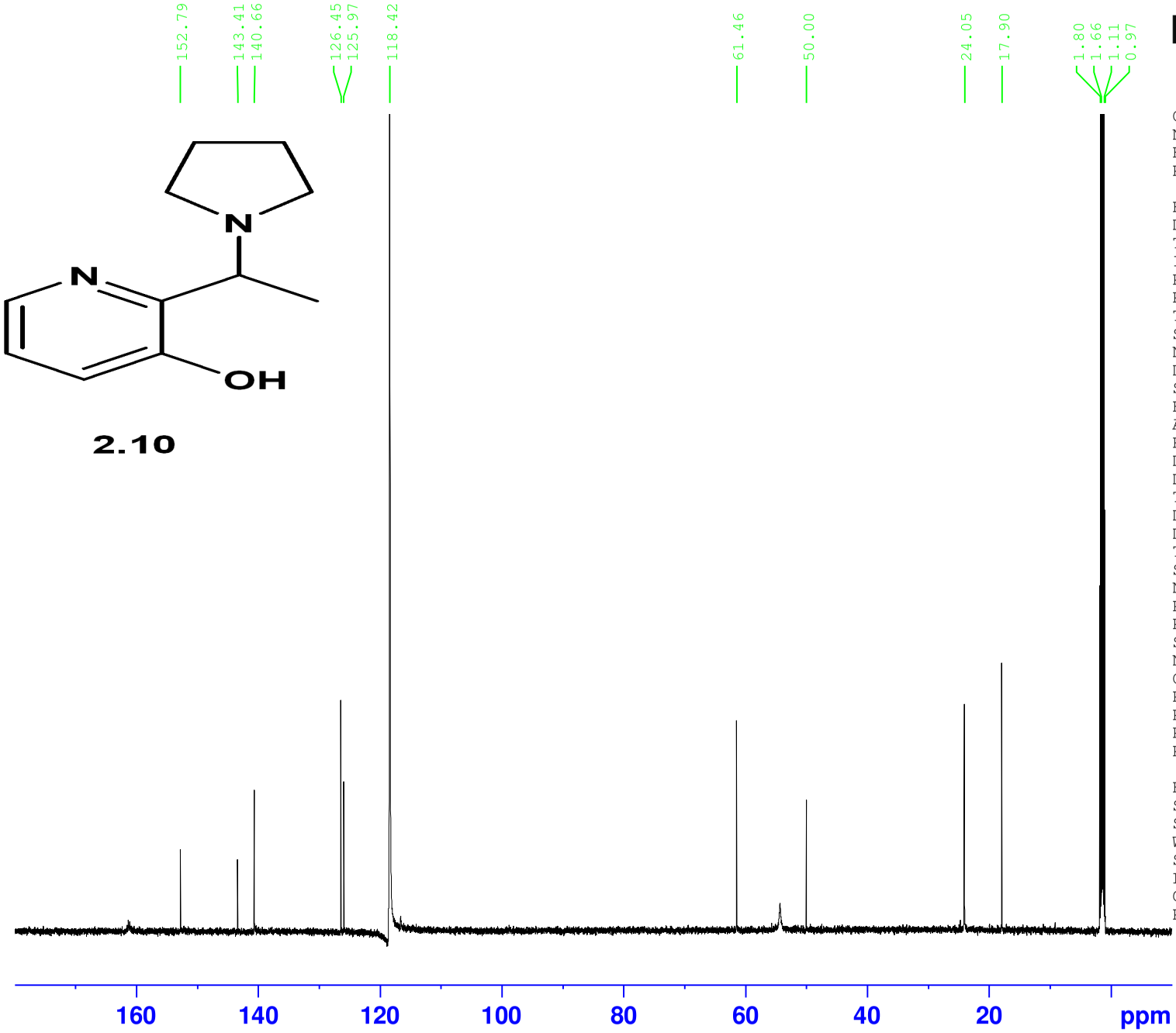
Faculty Hadad



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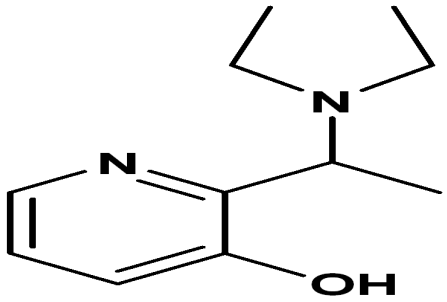
```

Faculty Hadad

8.294 8.292 8.286 8.284 7.627 7.625 7.613 7.611 7.482 7.474 7.468 7.460 5.088 5.076 5.065 5.054 3.489 3.482 3.472 3.464 3.462 3.448 3.438 3.424 3.419 3.412 3.308 3.147 3.141 3.058 2.123 2.111 2.107 2.103 2.099 2.095 2.091 1.734 1.723 1.432 1.420 1.408 1.392 1.279



2.11

Current Data Parameters
NAME JT-01-73-6
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20180220
Time 1.28 h
INSTRUM spect
PROBHD Z114607_0174 (
PULPROG zg30
TD 96152
SOLVENT CD3CN
NS 16
DS 2
SWH 12019.230 Hz
FIDRES 0.250005 Hz
AQ 3.9999232 sec
RG 104.7
DW 41.600 usec
DE 6.50 usec
TE 300.2 K
D1 1.00000000 sec
TD0 1
SFO1 600.0478245 MHz
NUC1 1H
P1 10.00 usec
PLW1 25.10000038 W

F2 - Processing parameters
SI 65536
SF 600.0449213 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

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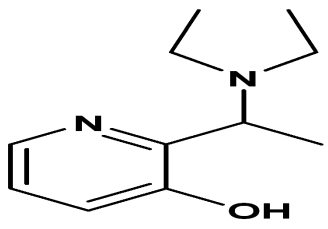

2.11


```
Current Data Parameters
NAME          JT-01-73-6
EXPNO                  2
PROCNO                 1

F2 - Acquisition Parameters
Date_           20180220
Time                2.20 h
INSTRUM            spect
PROBHD   Z114607_0174 (
PULPROG          zgpg30
TD                65536
SOLVENT           CD3CN
NS                 1024
DS                    4
SWH           36057.691 Hz
FIDRES         1.100393 Hz
AQ            0.9087659 sec
RG               189.17
DW               13.867 usec
DE                6.50 usec
TE                300.2 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                   1
SFO1        150.8965227 MHz
NUC1                13C
P1                9.40 usec
PLW1        78.16000366 W
SFO2        600.0474002 MHz
NUC2                1H
CPDPRG[2         waltz16
PCPD2             70.00 usec
PLW2        28.00000000 W
PLW12        0.57143003 W
PLW13        0.28742000 W

F2 - Processing parameters
SI                32768
SF          150.8813443 MHz
WDW                  EM
SSB       0
LB                1.00 Hz
GB        0
PC                1.40
```